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Electron Capture Gas Chromatographic Analysis of DDA: Utilization of 2-Chloroethanol Derivative

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The electron capture gas chromatographic analysis of DDA, the carboxylic acid metabolite of DDT, requires the formation of a suitable volatile derivative. The methyl ester has been utilized but had the unsatisfactory characteristic of poor electron capture response (1). This paper reports the utilization of the 2-chloroethanol derivative in the electron capture gas chromatographic analysis of DDA.

MATERIALS AND METHODS

Apparatus and Equipment: The gas chromatograph used was the Tracor Model MT-220 fitted with an electron capture detector system operated under the following conditions: a glass column, 6' x 1/4", packed with 1.95% QF-1/1.5% OV-17 on Supelcoport, 80/100 mesh; nitrogen carrier gas with a flow rate of 60 ml/min; column temperature, 200°C; injection port, 225°C; and detector temperature, 210°C.

Chromatographic Columns: Size 22, i.d. 7 mm, length 200 mm, Kontes No. 420100.

Standards: Analytical standard of 2,2-Bis(p-chlorophenyl) acetic acid (p,p'-DDA) 99+% was obtained from the pesticide repository of the Perrine Primate Laboratory, Perrine, Florida 33157.

Reagents: Hexane and Benzene, Mallinckrodt Nanograde solvents or equivalent. Boron trichloride-Methanol 10% BCl_3 (w/v) and Boron trichloride-2-chloroethanol 10% BCl_3 (w/v) were obtained from Applied Science Laboratory, State College, Pennsylvania.

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Silica gel, Woelm, Activity Grade I, Waters Associates, Inc., Framington, Mass. The adsorbent was dried for 48 hours at 170°C and stored in a dessicator. On the day of use, 1 gm of silica gel was deactivated by adding 15 microliters of water and 1 gm of silica gel to a 125 ml Erlenmeyer flask. The flask was stoppered and rotated until the water was evenly distributed throughout the adsorbent. The adsorbent was allowed to equilibrate for 2-3 hours with periodic shaking. The chromatographic column was prepared just before use (2).

Distilled water, sodium sulfate and glass wool, extracted with Nanograde hexane, were used throughout the experiment.

Esterification Procedure: To approximately 5 mg of p,p'-DDA in a test tube was added 2.0 ml of BCl_3-Methanol 10% w/v, or 2.0 ml of BCl_3-2-chloroethanol 10% w/v. Placed in a steam bath for thirty minutes, removed and quenched the reaction with 5 ml of cold distilled water. Extracted three times with 3 ml portions of hexane. Combined the hexane extracts and filtered through anhydrous sodium sulfate. Adjusted to appropriate volume for analysis by electron capture gas chromatography.

Preparation of Standard Solutions: A stock solution of p,p'-DDA in benzene was prepared by weighing 52 mg into a 50 ml volumetric flask. Diluted to volume with benzene. The methyl ester and the 2-chloroethanol derivatives was prepared from the stock solution by esterification with BCl_3-Methanol and BCl_3-2-chloroethanol, respectively. The concentration of the resulting working standards was 211 pg/µl and 64.4 pg/µl of the methyl ester and 2-chloroethanol derivatives, respectively, calculated as DDA.

Efficiency of Conversion of p,p'-DDA to the 2-Chloroethanol Derivative: To check the efficiency of conversion of p,p'-DDA to the 2-chloroethanol derivative, approximately 20 mg portions of p,p'-DDA were accurately weighed into each of six 50 ml test tubes (glass stoppered). The samples were esterified with 10 ml of BCl_3-2-chloroethanol. After esterification procedure, the hexane extracts, in previously tared concentrator tubes, were taken to dryness over a gentle stream of nitrogen. Concentrator tubes

were then placed in dessicator overnight to assure dryness and allow time to equilibrate. The concentrator tubes were then accurately reweighed to determine the weight of the 2-chloroethanol derivative of DDA. The efficiency of conversion of DDA to the 2-chloroethanol derivative was calculated.

Elution Pattern and Recovery of 2-Chloroethanol Derivative of DDA from the Silica Gel Column: Occasionally it is necessary to clean-up urine samples from the "general human population" prior to gas chromatographic determination utilizing the electron capture detector to remove interfering substances. The silica gel column chromatographic clean-up as described by Shafik, et al., (2) provided adequate clean-up of the 2-chloroethanol derivative of DDA prior to electron capture detection.

Duplicate samples of the 2-chloroethanol derivative of DDA (0.64 micrograms) were placed on silica gel columns and eluted with hexane followed by 10, 20, 30, 40, 50, 60, 70, and 80 percent benzene in hexane. Each sample was analyzed gas chromatographically to determine the elution pattern and percent recovery of the 2-chloroethanol derivative.

Response of 2-Chloroethanol and Methyl Ester Derivatives of DDA to Electron Capture Detection: To determine the sensitivity of the 2-chloroethanol derivative of DDA as compared with the sensitivity of the methyl ester derivative of DDA to electron capture detection; five injections, each containing 1.28 ng of the 2-chloroethanol derivative and 2.11 ng of the methyl ester derivative, were made into the gas chromatograph. The response was calculated for each derivative in terms of area under the curve and expressed as square millimeters per nanogram. The response ratio of 2-chloroethanol to the methyl ester was calculated.

RESULTS AND DISCUSSION

The efficiency of conversion of p,p'-DDA to the 2-chloroethanol derivative is shown in Table 1.

TABLE 1

EFFICIENCY OF CONVERSION OF p,p'-DDA TO THE 2-CHLOROETHANOL DERIVATIVE

No.	Weight of p,p'-DDA (mg.)	Weight of 2-Chloroethanol Derivative (mg)	% Conversion[a]
1	19.0	25.2	92.1
2	22.5	29.4	93.5
3	18.0	24.6	89.4
4	22.7	30.9	89.7
5	22.4	28.7	95.3
6	18.1	24.8	89.1
		Mean ± S.E. = 91.5 ± 1.0%	

[a]

$$\% \text{ Conversion} = \frac{342 \times \text{Weight of p,p'-DDA}}{280 \times \text{Wt. of 2-Chloroethanol Derivative}} \times 100.$$

The silica gel column chromatographic clean-up of the 2-chloroethanol derivative of p,p'-DDA was sufficient for the gas chromatographic analysis utilizing the electron capture detector.

The percent recovery of two samples, each containing 0.644 micrograms of p,p'-DDA after formation of the 2-chloroethanol derivative with BCl_3-2-chloroethanol 10% w/v from the silica gel column is given in Table 2.

TABLE 2

PERCENT RECOVERY OF 2-CHLOROETHANOL DERIVATIVE OF DDA FROM SILICA GEL COLUMN

Sample Number	2-Chloroethanol Derivative (as DDA) (µg)	% Recovery
1	0.624	96.9
2	0.629	97.7
	Mean ± S.E. = 97.3 ± 0.4%	

As shown in Table 2, the mean (±S.E.) for the recovery of the 2-chloroethanol derivative of DDA from the silica gel column was 97.3 + 0.4%. The column was eluted with hexane followed by TO, 20, 30, 40, 50, 60, 70, and 80 percent benzene in hexane. The 2-chloroethanol derivative was eluted in the 50% benzene in hexane fraction.

For future clean-up of the 2-chloroethanol derivative of DDA on the silica gel column it would be sufficient to elute with 40% benzene, 60% benzene, and 80% benzene in hexane with the 2-chloroethanol derivative appearing in the 60% benzene in hexane fraction.

The response of 2-chloroethanol and the methyl ester derivatives of DDA to the electron capture detector, as well as, the response ratio of 2-chloroethanol to the methyl ester is given in Table 3.

TABLE 3

RESPONSE OF 2-CHLOROETHANOL AND METHYL ESTER DERIVATIVES OF DDA TO ELECTRON CAPTURE DETECTION

Injection Number	Electron Capture Response (Peak Area)		Response Ratio 2-Chloroethanol Derivative / Methyl Ester Derivative
	2-Chloroethanol Derivative (mm^2/ng)	Methyl ester Derivative (mm^2/ng)	
1	293.65	72.96	3.69:1
2	278.52	76.78	3.63:1
3	293.75	75.59	3.89:1
4	266.41	75.35	3.54:1
5	284.18	74.64	3.81:1

The average electron capture response ratio of 2-chloroethanol derivative to the methyl ester derivative of DDA for the five determinations was 3.7 ± 0.1:1.

Another advantage of using the 2-chloroethanol derivative of DDA instead of the methyl ester derivative of DDA for the gas chromatographic analysis of DDA is that the retention time (Figure 1) of the 2-chloroethanol derivative is such that the DDA will be separated from other chlorinated hydrocarbons which might interfere with the analysis.

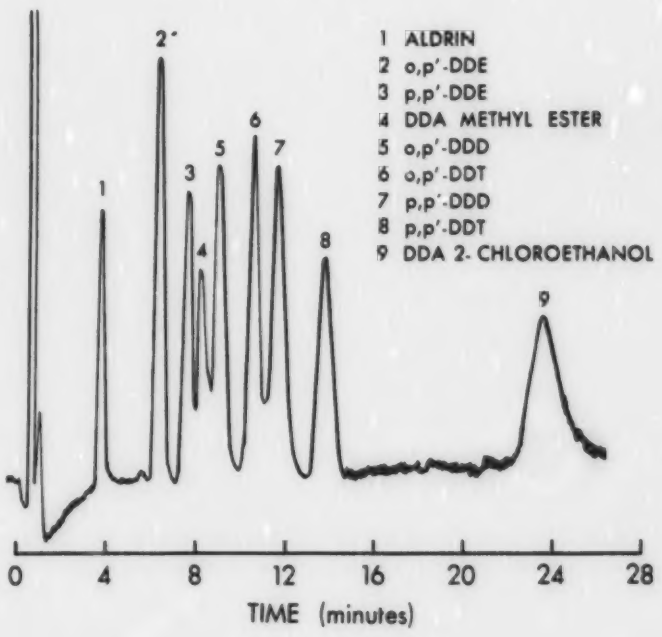

FIGURE 1 - Typical chromatogram of 9 organochlorine pesticides obtained using a 6' x 1/4" glass column packed with 1.95% QF-1/1.5% OV-17 on Supelcoport 80/100 mesh.

SUMMARY

This paper reports the use of the 2-chloroethanol derivative in the electron capture gas chromatographic analysis of DDA. The efficiency of conversion of DDA to the 2-chloroethanol derivative was 91.5 ± 1.0%. The utilization of a silica gel column for clean up prior to electron capture analysis is discussed and the elution pattern for the 2-chloroethanol derivative is given with a recovery of 97.3 ± 0.4%. The 2-chloroethanol derivative is 3.7 ± 0.1 times more responsive to electron capture detection than the methyl ester of DDA and produces a retention time which separates the DDA from other chlorinated hydrocarbon pesticides which might interfere with the analysis.

REFERENCES

1. CRANMER, M.F., et al., (1969). Bull. Environ. Contam. Toxicol. 4, 214-223.

2. SHAFIK, M.T., et al., (1971). Intern. J. Environ. Anal. Chem. 1, 23-33.

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A SENSITIVE GAS CHROMATOGRAPHIC METHOD FOR HUMAN CHOLINESTERASE DETERMINATION

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(Received February 8th, 1971)

SUMMARY

A rapid and sensitive method is described for the determination of cholinesterase. Plasma or red cells are incubated with 3,3-dimethylbutyl acetate as the substrate. The reaction product, 2,2-dimethyl butanol, is extracted into carbon disulfide and determined by GLC. The method is suitable for the analysis of samples that are quite low in enzyme activity.

INTRODUCTION

There are many useful and accurate methods for measurement of acetyl-cholinesterase[1-3]. However, to measure the effects of inhibitors on the cholinesterase of plasma and red cells, a rapid, accurate method is needed that requires only a few microliters of sample.

In several methods[4-6], enzyme activity is assayed by using 3,3-dimethylbutyl acetate (DMBA) as substrate. This paper presents a method in which the enzyme is incubated with the DMBA substrate and a buffer for a specified time (usually 30 min). The reaction is then stopped and the reaction product, 2,2-dimethyl-1-butanol (DMB) is extracted into carbon disulfide and measured by gas chromatography (GC). An additional advantage is that the CS_2 layer can be analyzed immediately or stored in the refrigerator for later analysis.

EXPERIMENTAL

Apparatus

A MicroTek MT-220 gas chromatograph equipped with a single hydrogen flame ionization detector and a Westronics dual pen recorder was used in this study.

The incubation of the reaction was carried out with a Thermolyne Dri-Bath permanently set at 37°. Components of the assay were added with Oxford pipettors and the reaction was carried out in a 5-ml glass-stoppered test tube. Blood samples were introduced into the reaction vessel either with Oxford pipettors or contained in heparinized capillary tubes.

Reagents

Reagents were obtained from the following sources: 3,3-dimethylbutyl acetate and 2,2-dimethylbutanol, K&K Laboratories; true cholinesterase, Type I, and pseudocholinesterase, Type IV, Sigma Chemical Co.; carbon disulfide, SpectrAR grade, Mallinckrodt Chemical Works; paraoxon, Farben Fabriken-Bayer-A.G.; diisopropylfluorophosphate, Aldrich Chemical Co.; Tween 20® emulsifier, Biscayne Chemical Laboratories; and control serum (Special Clinical Chemistry Control Serum), Hyland Laboratories. All other reagents were ACS grade.

Preparation of reagents

A stock solution of buffer is prepared by dissolving 44.73 g of potassium chloride, 4.12 g of sodium barbital, and 0.55 g of potassium monobasic phosphate in 200 ml of water[7]. The working solution is prepared by adding 20 ml of stock solution to 75 ml of water, adjusting the pH to 8.0 with 0.1 *N* HCl, and diluting the solution to a final volume of 100 ml with water.

To remove butanol impurities from DMBA, 5 parts of DMBA are mixed with 1 part of acetic anhydride. The mixture is kept at 37° for 24 h and then washed once with water to remove the acetic anhydride and impurities. A 0.2 *M* solution is prepared by diluting the DMBA with working buffer and adding emulsifier to a concentration of 0.2 %[8]. The pH is then adjusted to pH 8.0 with sodium hydroxide.

For gas chromatographic standards, DMB solutions containing 50, 100, 150, and 200 ng/μl are prepared in water containing 0.1 % emulsifier. 2 ml of CS_2 are added to 2 ml of each standard solution, and the mixtures are shaken well and centrifuged. The top layer is discarded and an appropriate volume of the CS_2 layer is injected into the gas chromatograph. Standard curves are prepared by plotting DMB concentration *vs.* peak height or peak area.

Concentrated formic acid (88 %) is diluted 1:1 with distilled water.

Chromatography

Column: a glass U-tube, 1/4 in. × 6 ft., was packed with Johns-Manville Chromosorb 101, mesh size 80–100, and conditioned overnight at 250°. Instrument conditions: column oven, 215°; detector, 265°; inlet, 245°; air, 300 ml/min; hydrogen, 20 ml/min; nitrogen, 40 ml/min.

Procedure

Pseudocholinesterase. To 1 ml of working buffer in a 5-ml ground glass-stoppered test tube, add 20 μl of plasma and warm to 37°. Add 0.2 ml of the 0.2 *M* DMBA solution and incubate for 30 min at 37°. Carry a blank, containing all reagents except plasma, throughout the procedure. After 30 min, stop the reaction with 0.1 ml of 44 % formic acid and then add 2 ml of CS_2. Shake the tube vigorously and centrifuge, and remove the top aqueous layer by aspiration. Inject an appropriate volume of the CS_2 into the gas chromatograph for DMB determination.

True cholinesterase. To 0.2 ml of water in a 5-ml ground glass-stoppered test tube, add 20 μl of packed red cells. Allow the cells to hemolyze, then add 0.5 ml of working buffer and warm to 37°. Proceed as in the determination for pseudocholinesterase, carrying through the procedure a blank containing all reagents except red cells.

Calculation

Compare the DMB concentration of the unknown samples, after subtracting the reagent blank, with standard curves determined each day with CS_2 extracts of the aqueous DMB standards. Calculate the results as μmoles of DMBA hydrolyzed per ml of plasma or red cells per min as follows:

$$\frac{\mu g \text{ DMB produced}}{\text{time of assay} \times \text{volume of plasma or red cells} \times 102\ \mu g/\mu mole}$$

RESULTS AND DISCUSSION

The pH of the reaction solution used for the more common substrates, *i.e.*, acetylcholine perchlorate and butyrylcholine chloride, has been 8.0. The optimum pH for the commercial pseudocholinesterase and the cholinesterases in Hyland control serum, human plasma, and red cells with DMBA as a substrate is between 7.5 and 8.0 (Fig. 1). A pH of 8.0 was selected as the most appropriate pH for the GLC method.

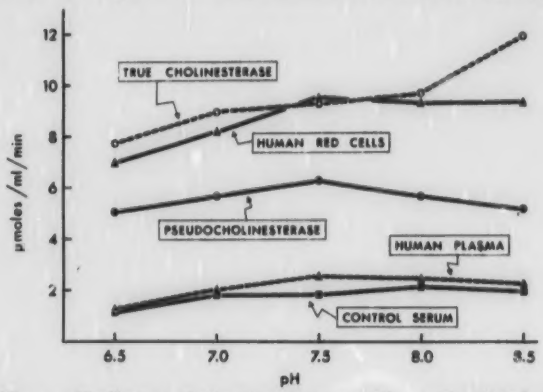

Fig. 1. pH effect on cholinesterase assay with 3,3-dimethyl butyl acetate as substrate.

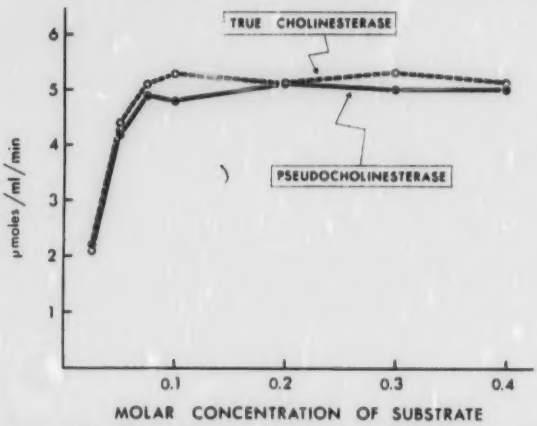

Fig. 2. Effect of substrate concentration on cholinesterase activity.

Fig. 2 shows the effect of substrate concentration on enzyme activity. At a DMBA concentration of 0.1 *M* the enzyme has reached peak activity; increased concentration of substrate does not inhibit either the true cholinesterase or pseudocholinesterase. A substrate concentration of 0.2 *M* was chosen to assure excess substrate during the entire reaction.

Varying dilutions of the commercial true cholinesterase and pseudocholinesterase were incubated with DMBA and the results were plotted as μmoles of DMB formed per ml of enzyme per min *vs.* relative concentration of enzyme present. Fig. 3 demonstrates that the sensitivity of the method is sufficient to yield linear results for enzyme activities down to 0.2 μmole/ml/min. As shown in Fig. 4, the reaction is linear when incubated for up to 50 min.

For the recovery studies, DMB was added to buffer and substrate in μg quantities so that the pH was not altered. After a 30-min incubation, formic acid and CS_2 were added as in the usual procedure. Table I shows that recovery of DMB from the CS_2 layer varied from 98 to 106 % over the concentration range examined.

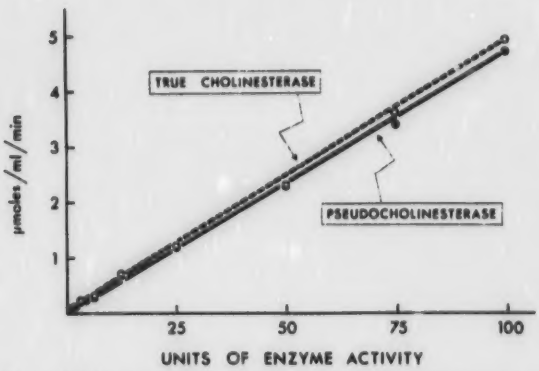

Fig. 3. Effect of enzyme concentration in the cholinesterase assay.

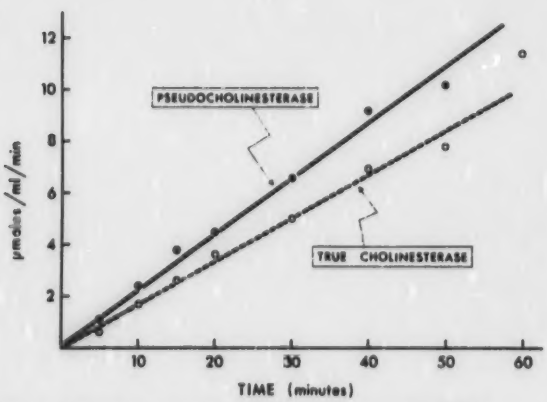

Fig. 4. Linearity of cholinesterase reaction with DMBA.

The reproducibility of the method is shown in Table II. Results of replicate analyses with the commercial true cholinesterase ranged from 5.4 to 5.6 μmoles/ml/min with a mean of 5.5 and a standard deviation of 0.08. Six analyses of the commercial pseudocholinesterase gave a range of 5.7 to 6.3 μmoles/ml/min with a mean of 6.0 and a standard deviation of 0.20.

Table III shows the results obtained with the present method on blood samples of the general population. Analyses of blood samples of 25 males and females gave a

TABLE I

RECOVERY OF 2,2-DIMETHYLBUTANOL FROM REACTION MIXTURE

2,2-Dimethylbutanol		*Recovery* (%)
Added (μg)	*Recovered* (μg)	
102	102	100
102	102	100
204	214	105
204	200	98
408	435	106
408	410	100

TABLE II

REPLICATE ANALYSES OF SINGLE SAMPLES

Sample	*μmoles of 3,3-DMBA hydrolyzed/ml/min*	
	True cholinesterase	*Pseudo-cholinesterase*
1	5.4	6.3
2	5.4	6.1
3	5.4	6.0
4	5.4	5.9
5	5.5	5.7
6	5.6	5.9
Mean	5.45	5.98
Standard deviation	0.08	0.20

TABLE III

CHOLINESTERASE VALUES OF GENERAL POPULATION

Sample	*Number of subjects*	*Mean 3,3-DMBA (μmoles/ml/min)*	*Standard deviation*	*Observed range (μmoles/ml/min)*
Plasma, males	18	3.4	0.6	2.5– 4.6
Plasma, females	7	2.9	0.5	2.1– 3.7
Plasma, combined results	25	3.2	0.6	2.1– 4.6
RBC, males	18	9.8	0.9	8.2–11.8
RBC, females	7	10.0	0.6	9.2–10.9
RBC, combined results	25	9.8	0.8	8.2–11.8

TABLE IV

COMPARISON OF GLC AND pH STAT DETERMINATION OF CHOLINESTERASE INHIBITION BY PARAOXON AND DIISOPROPYLFLUOROPHOSPHATE

Paraoxon (*μmoles*)	*Enzyme units*	*% Inhibition*		*Diisopropyl-fluorophosphate* (*μmoles*)	*Enzyme units*	*% Inhibition*	
		GLC	*pH Stat*			*GLC*	*pH Stat*
True cholinesterase							
5×10^{-5}	6.3	98	98	2×10^{-4}	3.1	87	91
2.5×10^{-5}	6.3	82	84	1×10^{-4}	3.1	61	65
1.2×10^{-5}	6.3	51	48	5×10^{-5}	3.1	38	35
6×10^{-6}	6.3	26	24	2.5×10^{-5}	3.1	18	17
r^a		0.9270	0.9190	r		0.9373	0.9718
Pseudocholinesterase							
4×10^{-4}	45	85	89	4×10^{-4}	22	74	79
2×10^{-4}	45	51	44	2×10^{-4}	22	38	41
1×10^{-4}	45	29	27	1×10^{-4}	22	23	19
5×10^{-5}	45	17	19	5×10^{-5}	22	9	10
r		0.9970	0.9971	r	22	0.9972	0.9995

[a] Correlation coefficient between cholinesterase inhibition and concentration of inhibitor.

range of 2.1 to 4.6 μmoles/ml/min for plasma cholinestrase, with a mean of 3.2 and a standard deviation of 0.6; for the red cell cholinesterase, the range was 8.2 to 11.8 with a mean of 9.8 and a standard deviation of 0.8. The relationship between the cholinesterase activities of human plasma and red cells as mesaured by GLC is similar but not identical to the relative activities as measured by the pH Stat method[2] (red cells 11.1–16.0; plasma 3.6–6.8).

For the inhibitor studies, the commercial enzymes were incubated at 37° with various concentrations of freshly prepared paraoxon or diisopropylfluorophosphate. At the end of 2 h, cholinesterase activity was determined; results are shown in Table IV. Absolute values for cholinesterase activity obtained by the pH Stat method[2] and this methods were not identical; however, the percent inhibition caused by the compounds compared very favorably, demonstrating a correlation between the two methods.

The cholinesterase values obtained by using Oxford pipettors were compared with those obtained by using the capillary tube technique[9]. The blood was collected in heparinized capillary tubes and spun in a micro hematocrit centrifuge. A 20-mm section containing plasma or red cells was cut from the capillary tubes and the entire section was added to the reaction vessel. The mean plasma pseudocholinesterase value with the capillary tube technique was 3.8 μmoles/ml/min (range 3.4–4.1; S.E. 0.09) and the mean red cell cholinesterase value was 14.1 (range 13.6–14.9; S.E. 0.15). The corresponding values obtained by using the Oxford pipettor were 3.5 and 14.0.

DMBA has been shown to be a suitable substrate for both true cholinesterase and pseudocholinesterase[4–6]. It is superior to acetylcholine and other commonly used substrates because its spontaneous decomposition and hydrolysis at the reaction pH are very low; in addition, both DMBA and DMB have good chromatographic qualities with Johns-Manville Chromosorb 101. DMBA has a retention time of about 6 min and can be used as an indicator of the presence of excess substrate at the end of

the reaction time. DMB, under the GC conditions described, has a retention time of approximately 4 min (Fig. 5).

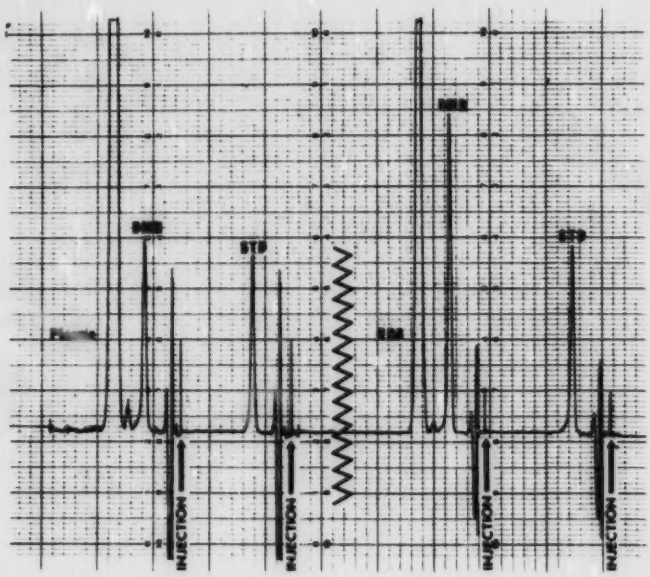

Fig. 5. Gas chromatogram of CS_2 layers from plasma and red cell cholinesterase assays.

Commercially available DMBA contains a small amount of DMB which interferes with the sensitivity of the method at very low activity. The reagent blank, also an indication of non-enzymatic hydrolysis of substrate, can be decreased by reacting the DMB with acetic anhydride to form DMBA. We found that the substrate remained stable at room temperature for about 2 months.

This method has been applied only to strong inhibitors of cholinesterase and not to reversible inhibitors.

REFERENCES

1 H. O. Michel, *J. Lab. Clin. Med.*, 34 (1949) 1564.
2 D. P. Nabb and F. Whitfield, *Arch. Environ. Health*, 15 (1967) 147.
3 E. R. Larson, *Microelectric Method for Blood Cholinesterase Determination*, Division of Laboratories, California State Department of Public Health, 1957.
4 D. H. Adams, *Biochim. Biophys. Acta*, 3 (1949) 1.
5 D. H. Adams and V. P. Whittaker, *Biochim. Biophys. Acta*, 3 (1949) 358.
6 L. A. Mounter and V. P. Whittaker, *Biochem. J.*, 47 (1950) 525.
7 R. J. Henry, *Clinical Chemistry: Principles and Techniques*, Hoeber Medical Division, New York, 1964, p. 494.
8 A. Todrick, K. P. Fellowes and J. P. Rutland, *Biochem. J.*, 48 (1951) 360.
9 F. D. Aldrich, G. F. Walker and C. A. Patase, *Arch. Environ. Health*, 19 (1969) 617.

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Analysis of *p,p'*-DDT and *p,p'*-DDE Pesticide Residues by Nuclear Magnetic Resonance Spectroscopy

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Nuclear magnetic resonance spectroscopy has been utilized for semiquantitative analysis and confirmation of mixtures of *p,p'*-DDT and *p,p'*-DDE. Several known mixtures have been analyzed as well as pesticide residues isolated from human adipose tissue of an occupationally exposed individual and animal liver tissue from rats dosed with *p,p'*-DDT. The relative error found for the mole fractions of *p,p'*-DDT and *p,p'*-DDE in standard mixtures of these materials ranged from 0.5 to 4.1%, depending on the relative concentrations of pesticides present. Gas chromatographic analyses performed on the tissue extracts by electron capture and electrical conductivity detection agreed well with the results obtained by nuclear magnetic resonance.

An analytical nuclear magnetic resonance (NMR) method has been applied to the confirmation and estimation of relative concentrations of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (*p,p'*-DDT) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p'*-DDE) isolated from adipose and liver tissue samples. Although a variety of chemical and instrumental methods has been utilized for the analysis of pesticide residues, simultaneous identification, quantitation, and confirmation of these materials present a difficult problem. The NMR technique, in principle, provides an excellent approach to trace organic compound analysis, since it allows quantitation and confirmation in a single instrumental procedure. However, because of the extremely low concentration levels of pesticide residues generally encountered and the inherent low sensitivity of the NMR technique, it cannot be routinely applied in securing analytical evidence for the presence of chlorinated pesticides in human and environmental media.

Thus, analytical verification and quantitation of organochlorine pesticides and their transformed metabolites are based on ultrasensitive procedures utilizing combinations of gas, ion exchange, paper, thin layer, and column chromatography (1). More definitive spectrophotometric procedures which have found application in residue analysis include infrared, fluorescent, phosphorescent, and ultraviolet methods (2), as well as mass spectrometry (3).

Although quantitative NMR methods are used routinely in analysis of drugs, food additives, and other industrially significant chemicals (4–6), no report is available regarding the utility for the analysis of pesticide residues isolated from human and animal tissues. NMR methods, of course, have been frequently and successfully applied in the structure elucidation of pesticide metabolites and other transformation products (7, 8).

This communication describes a brief study of the application of NMR to the semiquantitative analysis of mixtures of *p,p'*-DDT and *p,p'*-DDE isolated as residues from adipose tissue and liver tissue. Spectral data for a number of compounds of the DDT type have recently been published and the quantitative aspects of the NMR method, with examples, have been discussed (9). In the present case, the required sensitivity enhancement for examination of microgram quantities of pesticide residues was achieved by means of a time averaging computer. It was assumed and experimentally verified that sufficient pesticide material for NMR analysis could be obtained from tissues which would contain relatively high levels of pesticides, for example, adipose tissue of individuals with high exposure to pesticides and liver tissue from animals specifically dosed with pesticides.

Experimental

Materials

Standard solutions of the pesticides for the NMR analyses were prepared with 0.50 ml carbon tetrachloride containing 2% tetramethylsilane (TMS). The actual amounts of total *p,p'*-DDE and *p,p'*-DDT for each solution denoted in Table 1 ranged from 18 to 30 mg. The *p,p'*-DDE and *p,p'*-DDT (analytical grade materials of at least 99% purity as determined by electron capture gas-liquid chromatography (GLC)) were obtained from the Aldrich Chemical Co., Cedar Knolls, N.J.

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Analytical Procedure

Human adipose tissue of an individual occupationally exposed to DDT was obtained from the Dade County Medical Examiner's Office. The liver tissue was pooled from 2 rats that had been dosed with *p,p'*-DDT at a level of 15 mg/kg/day for 3 days. The procedure of Stanley and LeFavoure (10) with the addition of a final Florisil column cleanup step proved adequate to isolate the residues of *p,p'*-DDE and *p,p'*-DDT free of interfering materials.

The tissues (15 g quantities) were digested by a perchloric-acetic acid mixture, extracted into *n*-hexane, and chromatographed on a sulfuric acid-Celite column with *n*-hexane as eluant. The column filtrate and washings were concentrated to a volume of 0.5 ml and eluted through a micro-Florisil column (11). Three fractions were collected. Fraction 1, with 4 ml *n*-hexane as eluant, was discarded. Fraction 2, eluted with 8 ml *n*-hexane and 12 ml 1% (v/v) methanol in hexane, contained all of the *p,p'*-DDE and *p,p'*-DDT residues. Fraction 3, consisting of 12 ml 1% (v/v) methanol in hexane eluant, contained the more polar chlorinated pesticides and was not investigated further. Electron capture GLC was used to determine the residue contents of the individual fractions. The actual residue concentrations as determined by electron capture GLC were 4.32 ppm *p,p'*-DDT and 8.55 ppm *p,p'*-DDE for the adipose tissue, and 5.16 ppm *p,p'*-DDT and 3.22 ppm *p,p'*-DDE for the liver tissue. The eluate from fraction 2 was concentrated to near dryness by Kuderna-Danish type evaporative concentrators. The remaining traces of *n*-hexane were carefully removed with a stream of clean, dry nitrogen. The residue obtained was dissolved in 0.15 ml carbon tetrachloride containing 2% TMS. This solution was placed in an NMR sample microtube and was examined by NMR.

Instrumentation

The NMR spectra were recorded on a Varian Associates HA-100 high resolution spectrometer operating at 100 MHz. The line positions of the proton resonances were measured with a Hewlett Packard Model 5512 A electronic counter which displays the pen position in Hz relative to the internal TMS reference. Chemical shifts were measured to an estimated accuracy of ±0.2 Hz. Measurements were made at 36.2±1.0°C, the ambient temperature in the vicinity of the probe. A Varian C-1024 time averaging computer was used for continuous scanning and spectrum accumulation in the 700–800 Hz region. Integration of resonance peaks was done with a V-3521A integrator-decoupler, an integral unit of the HA-100 spectrometer.

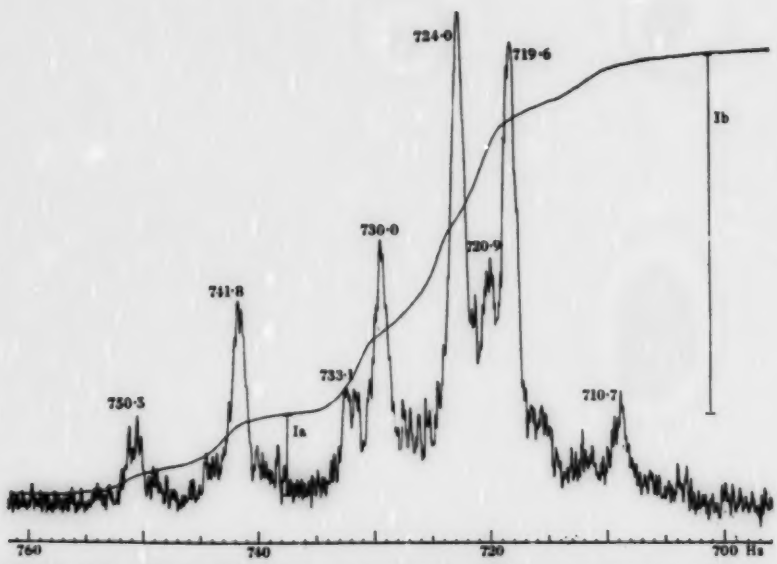

FIG. 1—100 MHz high resolution NMR spectrum of the aromatic protons of a mixture of *p,p'*-DDT and *p,p'*-DDE isolated from adipose tissue (250 time averaging computer scans).

Table 1. NMR and GLC analysis of p,p'-DDE and p,p'-DDT mixtures

Sample	Mole Fraction Added		Mole Fraction Found by NMR		Mole Fraction Found by Electron Capture GLC	
	p,p'-DDT	p,p'-DDE	p,p'-DDT	p,p'-DDE	p,p'-DDT	p,p'-DDE
Standard	0.817	0.183	0.809 (0.9)[a]	0.191 (4.1)[a]	0.811	0.189
Standard	0.692	0.308	0.684 (1.1)	0.316 (2.5)	0.685	0.315
Standard	0.599	0.401	0.607 (1.3)	0.393 (1.9)	0.595	0.405
Standard	0.473	0.527	0.466 (1.4)	0.534 (1.3)	0.478	0.522
Standard	0.348	0.652	0.353 (1.4)	0.647 (0.8)	0.344	0.656
Standard	0.152	0.848	0.156 (2.6)	0.844 (0.5)	0.151	0.849
Adipose tissue	—	—	0.305	0.695	0.312 (0.324)[b]	0.688 (0.676)[b]
Liver tissue	—	—	0.584	0.416	0.618	0.382

[a] Values in parentheses are the per cent relative error.
[b] These 2 values were obtained by means of GLC equipped with a Coulson electrical conductivity detector.

Results and Discussion

The NMR spectra of *p,p'*-DDT and *p,p'*-DDE have recently been discussed (9). For both *p,p'*-DDT and *p,p'*-DDE, the aromatic protons form 2 sets of 4 magnetically nonequivalent protons comprising an AA'BB' system. In the spectrum of *p,p'*-DDT the 2 most intense bands of the AA' portion are centered at 741.8 and 750.5 Hz and those for the BB' portion at 720.9 and 730.0 Hz. Similarly, for *p,p'*-DDE, the AA' transitions are found at 724.0 and 733.1 Hz and the BB' transitions at 710.7 and 719.6 Hz.

For the purpose of quantitation, the 2 intense peaks at 741.8 and 750.5 Hz for *p,p'*-DDT were chosen as the analytical peaks.

The mole fraction of *p,p'*-DDT in a mixture of *p,p'*-DDE and *p,p'*-DDT can be calculated by solving 2 simultaneous equations for x and y: $4x = Ia$ and $4x + 8y = Ib$. Ia and Ib are the integral values, measured in arbitrary units (Fig. 1); x and y are variables proportional to the number of moles of *p,p'*-DDT and *p,p'*-DDE, respectively; and the coefficients of x and y represent the number of protons responsible for the 2 integrals, Ia and Ib, in the spectrum.

The Ia integral results from the 4 AA' aromatic protons of *p,p'*-DDT and the Ib integral results from the 4 BB' aromatic protons of *p,p'*-DDT and the 8 aromatic protons of *p,p'*-DDE. The mole fraction, MF_{DDT}, of *p,p'*-DDT then is given by: $MF_{DDT} = x/(x + y)$; and the mole fraction, MF_{DDE}, of *p,p'*-DDE by: $MF_{DDE} = 1 - MF_{DDT}$. Synthetic mixtures were prepared with the concentrations given in Table 1. Spectra and integral data were obtained and the mole fraction of each component was determined as described above.

The residues of *p,p'*-DDT and *p,p'*-DDE isolated from the adipose tissue sample produced the spectrum illustrated in Fig. 1 after 250 scans of the time averaging computer. Calculated mole fractions for *p,p'*-DDT and *p,p'*-DDE were 0.305 and 0.695, respectively. Figure 2 presents the NMR spectrum of the mixture of the 2 pesticide residues isolated from liver tissue after 325 scans. In this case calculated mole fractions were 0.584 for *p,p'*-DDT and 0.416 for *p,p'*-DDE. The adipose tissue extract and liver tissue extract solutions were examined by electron capture GLC and, in the case of the adipose tissue, by GLC with electrical conductivity detection. The results of the analysis expressed in terms of the mole fraction of the individual pesticides are listed in Table 1 for comparison purposes. In both cases, the results agreed very well with those obtained by the NMR technique.

Table 1 summarizes the analytical data obtained. Also included are analytical results for several standard solutions of various known concentrations of *p,p'*-DDT and *p,p'*-DDE. Each result given, including those for the adipose tissue and liver tissue, was obtained from a single sample, but the integral data used were the average of at least 6 successive scans. The relative error found for the standard samples ranged from 0.5 to 4.1%. The accuracy of the results depends on the quantity of each component contained in the sample. The mixtures noted in Table 1 cover the range of relative concentration usually found for these 2 pesticides in tissue samples. The quantitative measurements become less accurate at the 2 extremes of this range.

These results show that NMR can be used to confirm the presence of and to semiquantitatively ascertain the relative composition of pesticide

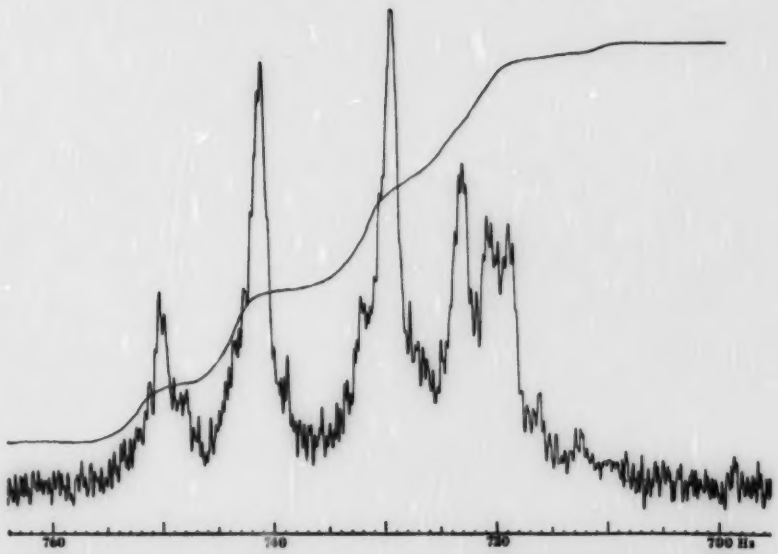

FIG. 2—100 MHz high resolution NMR spectrum of the aromatic protons of a mixture of p,p'-DDT and p,p'-DDE isolated from liver tissue (325 time averaging computer scans).

residues isolated from tissue samples. Because of the small amounts of materials isolated, the absolute quantities of pesticides present in the tissue were not determined. (An actual determination of the pesticide level may, however, be possible by dissolving an internal standard of known concentration in the final analytical solution.) Thus, the NMR method would be most useful for analysis of residues from tissues containing relatively high levels of materials as may be found in poisoning cases, high exposure cases, and metabolic studies in animals.

References

(1) *Pesticide Analytical Manual*, Vol. I, H. C. Barry, J. G. Hundley, and L. Y. Johnson (Eds.), Food and Drug Administration, Washington, D.C., 1966.

(2) Westlake, W. E., and Gunther, F. A., *Residue Rev.*, **18**, 175–217 (1967).

(3) Biros, F. J., and Walker, A. C., submitted to *J. Agr. Food Chem.*

(4) Slomp, G., Baker, R. H., Jr., and MacKellar, F. A., *Anal. Chem.* **36**, 375–380 (1964).

(5) Hollis, D. P., *Anal. Chem.* **35**, 1682–1684 (1963).

(6) Alexander, T. G., and Koch, S. A., *JAOAC* **50**, 676–678 (1967).

(7) Chen, P. R., Tucker, W. P., and Dauterman, W. C., *J. Agr. Food Chem.* **17**, 86–90 (1969).

(8) Belasco, I. J., and Reiser, R. W., *J. Agr. Food Chem.* **17**, 1000–1003 (1969).

(9) Keith, L. H., Alford, A. L., and Garrison, A. W., *JAOAC* **52**, 1074–1092 (1969).

(10) Stanley, R. L., and LeFavoure, H. T., *JAOAC* **48**, 666–667 (1965).

(11) Enos, H. F., Biros, F. J., Gardner, D. T., and Wood, J. P., presented at the 154th National Meeting of American Chemical Society, Sept. 10-15, 1967, at Chicago, Ill. (Abstract A-037).

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A Comparative Study of the Recovery of Metabolized Radiolabeled Pesticides from Animal Tissues

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INTRODUCTION

The importance of determining the efficiency of an analytical procedure for pesticide residue levels has long been recognized by residue chemists. Agricultural chemists in particular have expressed concern over the extraction efficiencies of solvents used in analysis of residue levels in plant materials.[1] The relationship between the amount of pesticide residues actually present in animal tissues and the amount extracted by a given analytical method is of equal importance and no systematic study has been reported in the literature. Biochemists investigating the metabolic pathways of radiolabeled pesticides in laboratory animals, however, frequently report extraction efficiencies for certain solvents and solvent systems as applied to the recoveries of radioactive materials from certain tissues of interest.[2]

The continuing development and evaluation of a microanalytical method[3] for the determination of pesticides and their metabolites in human and animal tissues prompted an investigation of the absolute recovery of these compounds. Dieldrin and p,p′-DDT were chosen for study because they represent the extremes of polarity of chlorinated hydrocarbon pesticides generally encountered in human and animal tissues. Experiments were conducted to provide information concerning (a) the absolute recovery of radiolabeled materials from the tissues examined and (b) the percentage of radioactivity in the tissue extract being due to free or gas chromatographable pesticide. A comparative study of the recovery of unlabeled pesticides from human tissue samples was also undertaken to validly relate the recovery of these materials using several solvents and solvent systems.

MATERIALS AND METHODS

The experimental animals (rabbits) were dosed intraperitoneally with 10 μc p,p′-DDT-^{14}C or dieldrin-^{14}C (Nuclear Research Chemicals, Inc., Orlando, Florida*) dissolved in glycerol/ethanol, on the first day of the experiment followed by a 10μc dose every five days until three doses had been administered. During the course of the experiment, 24-hour urine specimens were collected from each animal twice weekly. Five days following the last dose, the animal was sacrificed and specimens of blood, brain, liver, kidney, adipose tissue, adrenal, spleen and gonad were collected and stored at -10 C until the analysis was performed.

Analytical Procedure

The analytical procedure used has been described previously.[3] The extraction solvents employed were chloroform:methanol (1:1, v/v) for all tissues except blood, adipose tissue and urine. In the case of blood and adipose tissue, chloroform:methanol (2:1, v/v) and hexane, respectively, were used. For urine analysis, three analytical methods were employed as follows:

(a) Method W. Twenty-five ml of urine was taken to dryness on a rotary evaporator. The surface of the solution was sprayed with a small quantity of silicone defoamer (Sigma Chemical Co., St. Louis, Missouri) to minimize the foaming usually encountered when urine samples are concentrated. Four ml of methyl alcohol was then added to the residue. BCl_3 gas was bubbled into the flask for at least ten minutes or until the excess was seen pouring from the top of the flask. Ten ml of a 5% aqueous sodium sulfate solution was added to the reaction mixture followed by 4 ml of hexane. The solution was extracted a total of four times each with 4 ml of hexane. The extracts were combined, concentrated and analyzed for radioactivity content.

(b) Method C.[4] A 5 ml sample of urine was extracted three times with equal volume of 2% acetic acid in hexane. The extracts were combined and taken to dryness on a steam bath with a stream of dry air. A 5 ml ampoule of BF_3-methanol was then added to the residue. The sample was incubated at 50 C for 30 minutes. The reaction mixture was quenched with an equal volume of water. Finally, this solution was extracted four times with hexane. The combined extracts were then concentrated to a suitable volume and analyzed for radioactivity.

(c) Method B. This analytical procedure for urine has been described previously.[5]

The analytical results of the comparative recovery study for unlabeled materials in the human tissue samples were obtained using the procedure as described,[3] including a recent modification employing acetonitrile as extraction solvent. For radioactivity recovery studies, however, the cleanup steps involved in the procedure were eliminated since the presence of tissue co-extractives did not interfere in any way with the scintillation counting techniques used. Further, the efficiency of the analytical technique, per se, has been well established. The comparative data presented include recovery values for aldrin added to the tissue prior to analysis. In all cases the recoveries were excellent.

Radioactivity Analysis

Samples of whole tissue, extracts, and residues remaining after extraction were analyzed by scintillation counting techniques. Whole tissue and tissue residues were first combusted by the method described by Davidson and Oliverio.[6] The resultant ^{14}C labeled carbon dioxide was solubilized in scintillation solvent containing phenylethylamine and counted. The extraction efficiency was readily obtained by relating the radioactivity content of the extract to that present in the whole tissue prior to analysis. Control experiments were conducted utilizing tissues of animals that had not been dosed with radioactive pesticides. No interferences of quenching or chemiluminescence were encountered under the conditions of the experiments. Further, recovery studies were performed in which known quantities of radiolabeled p,p'-DDT were added to whole tissue prior to analysis. Combustions performed in this fashion indicated the efficiency of the radioactivity analysis to be 89.5-96.3%.

Thin Layer Chromatography

In order to determine the percentage radioactivity due to free, hence gas chromatographable, pesticide, the extracts from six tissue analyses (liver, kidney, brain, adipose tissue, blood and urine) including both p,p'-DDT-^{14}C and dieldrin-^{14}C dosage experiments were spotted on silica gel-G precoated thin layer chromatography (TLC) plates (Brinkmann Instruments, Inc., Westbury, N.Y.). The TLC plates were activated for two hours at 105 C just prior to analysis. The solvent system used for the elution of the pesticides was 3% acetone in hexane. Appropriate R_f values were determined for p,p'-DDT, p,p'-DDE, p,p'-DDD and dieldrin using unlabeled standard materials,[7] and the corresponding areas in the sample plates were removed and soaked in methanol overnight to dissolve the pesticides. The solutions were then filtered, concentrated to dryness, transferred to hexane solvent and counted by scintillation techniques. The radioactivity in the original sample extracts was then related to the quantity of radioactivity due to the eluted free pesticide to give the figures presented in Table 9.

RESULTS AND DISCUSSION

The results of the comparative study of pesticide recovery from blood are given in Table 1. Three solvents were examined for the efficiency study including hexane, chloroform:methanol (2:1, v/v), and acetonitrile. Recovery of added aldrin in all cases was good, ranging from 82-96%. The differences of up to 2 ppb in extracted pesticide for the three solvents are not considered to be significant for the purpose of this discussion. The average absolute recovery of radioactivity for blood is 71.4% for the DDT-dosed animal tissue and 75.1% for the

TABLE 1. Comparison of Extraction Solvents for Human Tissue (Blood)

Compound	*Gas Chromatographic Analysis* *Hexane (ppm)*	*Chloroform:Methanol (2:1) (ppm)*	*Acetonitrile (ppm)*
p,p'-DDE	.047 ± .001	.044 ± .002	.045 ± .002
p,p'-DDT	.027 ± .007	.026 ± .003	.0253 ± .0010
Heptachlor Epoxide	.0057 ± .0013	.0046 ± .0004	.0044 ± .0001
Dieldrin	.0081 ± .0015	.0071 ± .0004	.0059 ± .0001
Aldrin (% Recovery)	82 – 91	87 – 93	91 – 96

Absoulte Recovery of Radioactivity from Rabbitt Tissue (p,p'-DDT-^{14}C)

Sample	*DPM[a]/GM Rabbit Blood*	*DPM/GM Rabbit Blood Extract*	*DPM/GM Rabbit Blood Residue*	*%Recovery*
1	678	491	177	72.1
2	643	443	213	70.8

Absolute Recovery of Radioactivity from Rabbit Tissue (Dieldrin-^{14}C)

Sample	*DPM/GM Rabbit Blood*	*DPM/GM Rabbit Blood Extract*	*DPM/GM Rabbit Blood Residue*	*% Recovery*
1	273	208	71	76.4
2	289	213	85	73.9

[a] - disintegrations per minute

TABLE 2. Comparison of Extraction Solvents for Human Tissue (Liver)

Compound	*Gas Chromatographic Analysis Chloroform:Methanol (1:1) (ppm)*	*Acetonitrile (ppm)*
p,p′-DDE	1.53 ± .06	1.48 ± .05
p,p′-DDT	0.22 ± .01	0.24 ± .01
p,p′-DDD	0.35 ± .02	0.33 ± .02
Heptachlor Epoxide	0.067 ± .006	0.067 ± .005
Dieldrin	0.120 ± .004	0.14 ± .004
Aldrin (% Recovery)	75 – 89	87 – 98

Absolute Recovery of Radioactivity from Rabbit Tissue (p,p′-DDT-^{14}C)

Sample	*DPM/GM Rabbit Liver*	*DPM/GM Rabbit Liver Extract*	*DPM/GM Rabbit Liver Residue*	*% Recovery*
1	4154	3188	898	76.7
2	4067	3035	925	74.6

Absolute Recovery of Radioactivity from Rabbit Tissue (Dieldrin-^{14}C)

Sample	*DPM/GM Rabbit Liver*	*DPM/GM Rabbit Liver Extract*	*DPM/GM Rabbit Liver Residue*	*% Recovery*
1	6879	5407	1589	78.6
2	6754	5361	1401	79.4

dieldrin-dosed animal tissue. These relatively low recovery values reflect the considerable conjugation and binding that must occur in blood resulting in somewhat incomplete extraction of pesticide under the conditions of our analytical method. The low radioactivity content of the blood of the dieldrin-dosed rabbits as compared to the DDT-dosed rabbits is worthy of comment and indicates that dieldrin is eliminated via the bile and the intestine with greater facility than DDT. This observation is further substantiated by the relatively low radioactivity content of the urine (Table 5), kidney (Table 6), and gonad (Table 8) of the dieldrin-dosed rabbit tissues examined when compared with the DDT-dosed animal tissues.[8]

Recoveries of pesticides from liver tissue employing chloroform:methanol (1:1, v/v) and acetonitrile are given in Table 2. The comparison is favorable for all five pesticides found. In this case the recovery of added aldrin ranged from 75% using chloroform: methanol to 98% using acetonitrile as extraction solvent. The recovery of radioactivity employing chloroform:methanol as

TABLE 3. Comparison of Extraction Solvents for Human Tissue (Brain)

Compound	*Gas Chromatographic Analysis Chloroform:Methanol (1:1) (ppm)*	*Acetonitrile (ppm)*
p,p′-DDE	.095 ± .004	.092 ± .005
p,p′-DDT	.056 ± .005	.062 ± .003
Heptachlor Epoxide	.0016 ± .0006	.0020 ± .0006
Dieldrin	.0020 ± .0008	.0040 ± .0004
Aldrin (% Recovery)	84 – 88	94 – 102

Absolute Recovery of Radioactivity from Rabbit Tissue (p,p′-DDT-^{14}C)

Sample	*DPM/GM Rabbit Brain*	*DPM/GM Rabbit Brain Extract*	*DPM/GM Rabbit Brain Residue*	*% Recovery*
1	458	393	63	85.8
2	497	418	75	84.1

Absolute Recovery of Radioactivity from Rabbit Tissue (Dieldrin-^{14}C)

Sample	*DPM/GM Rabbit Brain*	*DPM/GM Rabbit Brain Extract*	*DPM/GM Rabbit Brain Residue*	*% Recovery*
1	564	511	67	90.7
2	578	510	79	88.5

TABLE 4. Comparison of Extraction Solvents for Human Tissue (Adipose Tissue)

Compound	Gas Chromatographic Analysis Hexane (ppm)	Acetonitrile
p,p'-DDE	3.9 ± .05	4.3 ± 0.3
p,p'-DDT	0.79 ± 0.10	0.97 ± 0.02
p,p'-DDD	0.025 ± 0.005	<02
Heptachlor Epoxide	0.35 ± 0.12	0.25 ± 0.60
Dieldrin	0.15 ± 0.02	0.12 ± 0.05
Lindane	0.07 ± 0.008	0.05 ± 0.01
Aldrin (% Recovery)	61 - 72	82 - 92

Absolute Recovery of Radioactivity from Rabbit Tissue (p,p'-DDT-^{14}C)

Sample	DPM/MGM Rabbit Adipose Tissue	DPm/MGM Rabbit Adipose Tissue Extract	DPM/MGM Rabbit Adipose Tissue Residue	% Recovery
1	108	99	–	91.6
2	125	118	–	94.4

Absolute Recovery of Radioactivity from Rabbit Tissue (Dieldrin-^{14}C)

Sample	DPM/MGM Rabbit Adipose Tissue	DPM/MGM Rabbit Adipose Tissue Extract	DPM/MGM Rabbit Adipose Tissue Residue	% Recovery
1	84	81	–	96.4
2	89	85	–	93.7

extraction solvent was 75.6% for DDT and 79.0% for dieldrin. As in the blood extractions, the radioactivity recovery is somewhat low indicating the high degree of enzymatic conjugation that occurs in the liver. Since the liver is the primary site of metabolism, the concentration of polar metabolic products both free and conjugated would be high, thus the extraction efficiency would be lower.

Table 3 presents the recovery data obtained for brain tissue. The relative efficiencies for chloroform:methanol and acetonitrile compare favorably. Recoveries of added aldrin ranged from 84-102%. The absolute radioactivity recovery for DDT was 84.9%, and for dieldrin, 89.6%, considerably better than for the liver and blood tissues discussed earlier. Some storage and little metabolic processes would occur in the brain and consequently recoveries of pesticides would be very good.

The data obtained in the recovery study for adipose tissue are given in Table 4. The comparative study indicates that hexane and acetonitrile as solvents compare very well in efficiency for recovering the six pesticides found by gas chromatography. In addition, aldrin recoveries, ranging from 61% to 92% were found acceptable. Since the adipose tissue is the primary site of pesticide storage, the recoveries of pesticides employing the analytical procedure described, should be very good. This was found to be the case as the recovery of radioactivity for DDT averaged 93.0% and for dieldrin, 95.1%.

The absolute recovery of radioactivity from urine is given in Table 5. The results employing Method C (see the Materials and Methods section) show an average extraction efficiency of 63.6%. For Method W, the extraction efficiency averaged 72.1%. The average extraction efficiency for Method B was found to be 70.9%. The relatively low recoveries found for urine indicate that a high percentage of excreted material is present in a highly polar and/or conjugated form.

Table 6 presents data obtained for the recovery study performed on kidney tissue. The comparative data show an approximately equal extraction efficiency for chloroform:methanol (1:1, v/v) and acetonitrile. Aldrin recoveries as indicated in the table ranged from 76% to 95%. The recovery of radioactivity from DDT-dosed animal tissue was an average of 71.2% and for the dieldrin-dosed animal tissue, 90.2%. The rather large difference in recovery for these two pesticides cannot be accounted for at this time. The fact that the recovery of dieldrin is high obviously reflects a somewhat diminished degree of conjugation and binding of this pesticide and its metabolites as compared to DDT. The question of whether this finding is related to the relative degrees of fecal and urinary excretion of these two pesticides remains to be answered.

The data for the recovery of pesticide from adrenal tissue are given in Table 7. Comparative recoveries for chloroform:methanol and acetonitrile compare very well. Recoveries of added aldrin were excellent ranging from

TABLE 5. Recovery of Radioactivity from Rabbit Urine (p,p'-DDT-^{14}C)

Gas Chromatographic Analysis

I. Method C.

Sample	*DPM/ML Rabbit Urine*	*DPM/ML Rabbit Urine Extract*	*DPM/ML Rabbit Urine Residue*	*% Recovery*
1	1410	828	515	61.7
2	1284	760	446	63.0
3	2529	1745	885	66.3

II. Method W.

Sample	*DPM/ML Rabbit Urine*	*DPM/ML Rabbit Urine Extract*	*DPM/ML Rabbit Urine Residue*	*% Recovery*
1	1410	976	386	71.6
2	1284	859	339	71.7
3	2529	1706	634	72.9

Recovery of Radioactivity from Rabbit Urine (Dieldrin-^{14}C)

III. Method B.

Sample	*DPM/ML Rabbit Urine*	*DPM/ML Rabbit Urine Extract*	*DPM/ML Rabbit Urine Residue*	*% Recovery*
1	165	115	78	70.5
2	173	124	63	71.3

TABLE 6. Comparison of Extraction Solvents for Human Tissue (kidney)

Compound	*Gas Chromatographic Analysis Chloroform:Methanol (1:1) (ppm)*	*Acetonitrile (ppm)*
p,p'-DDE	0.89 ± .03	.084 ± .04
p,p'-DDT	0.17 ± .01	0.19 ± .02
p,p'-DDD	0.22 ± .06	0.27 ± .05
Heptachlor Epoxide	0.042 ± .005	0.038 ± .004
Dieldrin	0.093 ± .007	0.095 ± .003
Aldrin (% Recovery)	76 - 83	88 - 95

Absolute Recovery of Radioactivity from Rabbit Tissue (p,p'-DDT-^{14}C)

Sample	*DPM/GM Rabbit Kidney*	*DPM/GM Rabbit Kidney Extract*	*DPM/GM Rabbit Kidney Residue*	*% Recovery*
1	2683	1941	656	72.3
2	2367	1661	685	70.2

Absolute Recovery of Radioactivity from Rabbit Tissue (Dieldrin-^{14}C)

Sample	*DPM/GM Rabbit Kidney*	*DPM/GM Rabbit Kidney Extract*	*DPM/GM Rabbit Kidney Residue*	*% Recovery*
1	482	436	52	91.1
2	503	449	67	89.4

TABLE 7. Comparison of Extraction Solvents for Human Tissue (Adrenal)

Compound	Gas Chromatographic Analysis Chloroform:Methanol (1:1) (ppm)	Acetonitrile (ppm)
p,p′-DDE	0.087 ± .003	0.081 ± .002
p,p′-DDT	0.023 ± .005	0.031 ± .003
Heptachlor Epoxide	0.0011 ± .0004	0.0016 ± .0008
Dieldrin	0.0036 ± .0006	0.0032 ± .0004
Aldrin (% Recovery)	86 — 89	92 — 98

Absolute Recovery of Radioactivity from Rabbit Tissue (p,p′-DDT-^{14}C)

Sample	DPM/GM Rabbit Adrenal	DPM/GM Rabbit Adrenal Extract	DPM/GM Rabbit Adrenal Residue	% Recovery
1	1163	987	188	85.4
2	1097	946	163	87.2

Absolute Recovery of Radioactivity from Rabbit Tissue (Dieldrin-^{14}C)

Sample	DPM/GM Rabbit Adrenal	DPM/GM Rabbit Adrenal Extract	DPM/GM Rabbit Adrenal Residue	% Recovery
1	455	404	61	88.9
2	468	429	48	91.3

TABLE 8. Comparison of Extraction Solvents for Human Tissue (Gonad)

Compound	Gas Chromatographic Analysis Chloroform:Methanol (1:1) (ppm)	Acetonitrile (ppm)
p,p′-DDE	0.024 ± .0003	0.032 ± .0008
p,p′-DDT	0.016 ± .0002	0.021 ± .0004
Heptachlor Epoxide	0.0042 ± .0005	0.0038 ± .0001
Dieldrin	0.0037 ± .0001	0.0041 ± .0003
Aldrin (% Recovery)	81 - 88	86 - 93

Absolute Recovery of Radioactivity from Rabbit Tissue (p,p′-DDT-^{14}C)

Sample	DPM/GM Rabbit Gonad	DPM/GM Rabbit Gonad Extract	DPM/GM Rabbit Gonad Residue	% Recovery
1	1236	1117	123	89.4
2	1368	1228	149	88.7

Absolute Recovery of Radioactivity from Rabbit Tissue (Dieldrin-^{14}C)

Sample	DPM/GM Rabbit Gonad	DPM/GM Rabbit Gonad Extract	DPM/GM Rabbit Gonad Residue	% Recovery
1	389	367	24	94.3
2	415	390	38	94.1

TABLE 9. Percent Free Pesticide Present in p,p′-DDT-^{14}C and Dieldrin-^{14}C Dosed Rabbit Tissues

Tissue Sample	% Free Pesticide[a,b] (p,p′-DDT-^{14}C Dose)	% Free Pesticide[a,b] (Dieldrin-^{14}C Dose)
Liver	61.6[d]	67.8[d]
Kidney	70.8	72.3
Brain	81.8	85.3
Adipose Tissue	92.0	94.7
Blood	67.9	63.9
Urine	56.3[e]	68.4[e]

[a]Determined by Scintillation Counting Techniques.
[b]Percent p,p′-DDE, p,p′-DDT and p,p′-DDD as determined by Thin Layer Chromatography.
[c]Percent Dieldrin as determined by Thin Layer Chromatography.
[d]Average of two determinations.
[e]The analytical procedure used for urine was Method B.

86–98%. The absolute recovery of radioactivity data shows an efficiency of 86.3% for DDT and 90.1% for dieldrin. Finally, Table 8 presents similar data obtained for gonad tissue. Recoveries of the four pesticides found were equal employing either chloroform:methanol or acetonitrile as extraction solvent. Further, aldrin recoveries were good ranging from 81–93%. The recovery of radioactivity was found to be an average of 89.0% for DDT and an average of 94.2% for dieldrin. Not included in the tables was an extraction efficiency obtained for dieldrin from rabbit spleen tissue. Using chloroform:methanol (1:1, v/v) as extraction solvent, the efficiency was found to be 91.3% and 92.5% for two determinations.

The data in Table 9 illustrate the percentage of radioactivity present in the tissue extracts due to free pesticides and/or metabolites. For the p,p′-DDT-^{14}C-dosed rabbit tissues the percentage of free pesticide refers to total radioactivity due to p,p′-DDT, p,p′-DDE and p,p′-DDD. For the dieldrin-^{14}C-dosed rabbit tissues the percentage of free pesticide refers to radioactivity due only to dieldrin. The results reflect the relative degree of storage and metabolism occurring in the six tissues examined. For example, the percentage of free pesticide found in brain and adipose tissue is high both for the p,p′-DDT-^{14}C-dosed rabbit tissue and the dieldrin-^{14}C-dosed rabbit tissue. Values for free pesticide in the extracts are 81.8% and 92.0%, respectively, for brain and adipose tissue in the DDT-dosed rabbit tissues and 85.3% and 94.7%, respectively, for brain and adipose tissue in the dieldrin-dosed rabbit tissues. The relatively low value for urine (56.3%) of the DDT-dosed animals reflects the fact that the p,p′-DDA-^{14}C was not accounted for by the TLC analysis. The value for percentage of free pesticide in the dieldrin-dosed rabbit urine was 68.4%. Other values as listed in the Table are: liver 61.6%, kidney 70.8%, and blood 67.9% for the DDT-dosed animal tissues. For the dieldrin-dosed animal tissue values for percentage of free pesticide are as follows: liver 67.8%, kidney 72.3%, and blood 63.9%.

Note Added in Proof

A recent investigation[9] reports that recoveries of metabolically incorporated dieldrin-^{14}C obtained from several pheasant tissues were as follows: 99% for adipose tissue, 91% for liver, 90% for eggs, and 86% for feces. The method used for adipose tissue was essentially that reported in this investigation, i.e., a hexane extraction. The reported method for liver, eggs and feces was a combination extraction-cleanup using a florisil column and 20% v/v dichloromethane in light petroleum. The results showed that recoveries of dieldrin were similar for metabolically incorporated and added insecticide and that the results by gas-liquid chromatography are similar to those obtained by scintillation counting. It was concluded by these authors that metabolically incorporated dieldrin is efficiently extracted from several pheasant tissues by the techniques used.

CONCLUSIONS

The recovery data presented in this investigation illustrates the comparatively good efficiency of selected solvents for extracting certain pesticides and their metabolites from both human and animal tissues. The initial objective outlined in the introduction has been accomplished and the data presented should lend some degree of confidence to values of residue levels reported in various tissues employing the extraction and cleanup techniques as described. Some shortcomings of the recovery study may be pointed out. The data described were obtained using animal tissues and thus may not be applied without certain reservations to human tissue. In addition, the metabolized material extracted from the rabbit tissue is on the basis of a short-term exposure to these materials. Human exposure to pesticides, on the other hand, occurs on a long-term basis. Thus the metabolism mechanism including storage, transport, and excretion may differ for these two cases resulting in somewhat varied recoveries of pesticides. Finally, the absolute recovery data are presented on the assumption that no in vivo transformations to insoluble polar metabolites occur. This latter assumption has been partially circumvented for the six tissues listed in Table 9 by the TLC investigation of free pesticide in the tissue extracts.

Furthermore, the TLC recovery figures reported in Table 9 show that the recovery of free gas chromatographable insecticidal material both unchanged and metabolized is quantitatively excellent. Since the unrecovered material is of a highly polar nature and not easily eluted from the origin by the elution solvents used, it would most certainly not be detected by gas-liquid chromatography.

ACKNOWLEDGMENT: The authors wish to express their gratitude to Mr. William Oller for his assistance with the animal dosing experiments and radioactivity analyses.

REFERENCES

1 Wheeler, W.B., and Frear, D.E.H.: Extraction of chlorinated hydrocarbon pesticides from plant materials, in *Residue Reviews*, F. A. Gunther, ed., 16:86-102, 1966. New York: Springer Verlag, (and references cited herein).

2 See for example, the following references:
(a) Grover, P.L., and Sims, P.: The metabolism of lindane in rats. *Biochem. J.* 96:521-525, 1965
(b) Ludwig, G., Weis, J., and Korte, F.: Metabolism of insecticides. VII. Excretion and distribution of aldrin-^{14}C and its metabolites after oral administration for a long period of time, *Life Sci.* 3:123-130, 1964
(c) Poonawalla, N.H., and Korte, F.: Excretion, distribution and metabolism of α-chlordane-^{14}C by rats, *Life Sci.* 3:1497-1500, 1964

3 Enos, H.F., et al.: Micromodification of the Mills Procedure for the Detection of Pesticides in Human Tissues, *Abstracts, 154th Meeting, Am. Chem. Soc.*, Chicago, Ill., Sept. 1967, A-43

4 Cranmer, M.F., Carroll, J.J., and Copeland, M.F.: Determination of DDT and metabolites, including DDA, in human urine by gas chromatography, *Bull. Environ. Contamin. Toxicol.* 4:214-233, 1969

5 Cueto, C., Jr., and Biros, F. J.: Chlorinated insecticides and related materials in human urine, *Toxic. Appl. Pharmacol.* 10:261-269, 1967

6 Davidson, J. D., and Oliverio, V. T.: Tritium and carbon-14 by oxygen flask combustion, *Atomlight* No. 60, May, 1967, New England Nuclear Corp., Boston, Mass. See also: *Anal. Biochem.* 4:188-189, 1962

7 Kovacs, M. F., Jr.: Thin layer chromatography for pesticide residue analysis, *J. Assoc. Off. Anal. Chem.* 48:1018-1022, 1965

8 For a general review of the metabolism, storage and excretion of chlorinated hydrocarbon insecticides see: Hayes, W. J., Jr.: Review of the metabolism of chlorinated hydrocarbon insecticides especially in mammals, *Ann. Rev. Pharm.*, W. C. Cutting, ed., 5:27-52, 1965. Palo Alto, Calif.

9 Greichus, Y., Lamb, D., and Garrett, C.: Efficiency of extraction of metabolically incorporated HEOD (carbon-14) from pheasant tissues, eggs and faeces, *Analyst* 93:323-325, 1968

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PESTICIDE
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Pesticide Residue Analysis in Human Tissue by Combined Gas Chromatography–Mass Spectrometry

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Pesticide residues in human adipose tissue and liver tissue samples were identified by mass spectrometry coupled with gas chromatography. An extensive extraction and cleanup procedure was adapted from existing analytical methods to separate pesticide residues from the tissue samples. Concentration of pesticides in tissue ranged from 0.073 to 28.7 ppm. Mass spectral data were computer-enhanced to identify heptachlor epoxide. Other pesticides confirmed included the β-isomer of 1,2,3,4,5,6-hexachlorocyclohexane (HCH), several isomers and metabolites of *p,p'*-DDT, and dieldrin. Advantages, limitations, and suggested modifications for improving the combined gas chromatography—mass spectrometry technique for pesticide residue analysis are discussed.

Mass spectrometry coupled with gas chromatography is a particularly useful approach to the analysis of pesticide residues and their metabolites because positive identification of the components of a mixture can be made without prior separation and at a sensitivity compatible with the limited quantities of residues encountered. Mass spectrometry is widely employed for analysis of individual residues and metabolites isolated by conventional separation techniques such as thin-layer chromatography, liquid chromatography, paper and column chromatography, and gas-liquid chromatography; yet few reports can be found in the literature on the utility of the combined gas chromatography—mass spectrometry method for residue analysis.

Several specialized techniques are available for efficient trapping and collection of individual gas chromatographic peaks for subsequent mass spectral analysis by the direct probe introduction method (Amy *et al.*, 1965; Damico *et al.*, 1967; Muller, 1963; Mumma and Kantner, 1966). These methods, although practical and reliable, are time-consuming and may risk sample loss by oxidation or hydrolysis. Amy *et al.* (1965) previously listed a number of disadvantages of the direct technique, such as memory effects associated with molecular separators and ion source pressure fluctuations due to flow of carrier gas into the mass spectrometer system. These disadvantages, however, are no longer a serious problem in view of the improvements made in commercially available instruments. In addition, decomposition of the components of gas chromatographic effluents can be minimized by conversion to all-glass inlet systems or by use of glass capillary columns of sufficiently small inner diameter to allow direct entry to the ion source (McFadden, 1966).

Recent reports have focused attention on the need for confirming pesticide residues, particularly those isolated from environmental samples (Elgar, 1967; Schechter, 1968) and for chemical identity in ultramicroanalysis in general (Robinson *et al.*, 1966). In this area the combined technique of gas chromatography–mass spectrometry offers many advantages over other forms of spectroscopic techniques and physical measurements. These advantages include an increase in the speed of analysis, elimination of the necessity for isolating minute quantities of pure sample, and greater certainty in identification of the eluted component than that achieved by gas chromatographic retention times alone (Watson and Biemann, 1965), an advantage of particular importance for residue confirmation. Mass spectrometry allows direct determination of molecular weight and, frequently, the number and kinds of heteroatoms present in the molecule. Additionally, full identification and structural information can be obtained by a thorough analysis of the spectrum.

This communication reports the practical application of gas chromatography–mass spectrometry to residue analysis of several chlorinated hydrocarbon residues in human tissue samples. The mass spectrum of each pesticide eluted from the gas chromatographic column was obtained and compared with those available in the literature (Damico *et al.*, 1968; Jörg *et al.*, 1966; Kantner and Mumma, 1966) or from standard pesticidal compounds. These pesticides all gave easily recognizable molecular ion peaks and characteristic ion fragments. The number of chlorine atoms in each fragment ion, including the parent ion, was readily determined by the specific isotopic distribution pattern (Beynon, 1960). Initially, the objectives of this endeavor were to determine which available analytical procedure offered the best approach to sample extraction and cleanup with minimum gas chromatographic and mass spectrometric interferences; to ascertain the lower practical limit of identification of pesticide residues; and to establish the ability of the combined gas chromatograph-mass spectrometric method to distinguish among configurational isomers (*e.g.*, the isomers of 1,2,3,4,5,6-hexachlorocyclohexane, HCH) and structural isomers [*e.g.*, 1,1-bis-(*p*-chlorophenyl)-2,2,2-trichloroethane and 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane; *p,p'*-DDT and *o,p'*-DDT, respectively].

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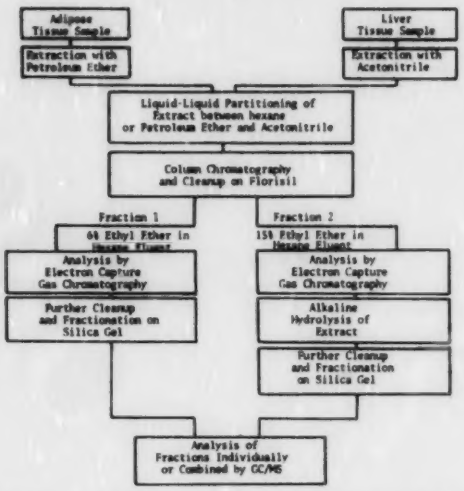
Adipose Tissue Sample
Liver Tissue Sample
Extraction with Petroleum Ether
Extraction with Acetonitrile
Liquid-Liquid Partitioning of Extract between hexane or Petroleum Ether and Acetonitrile
Column Chromatography and Cleanup on Florisil
Fraction 1
Fraction 2
6% Ethyl Ether in Hexane Eluent
15% Ethyl Ether in Hexane Eluent
Analysis by Electron Capture Gas Chromatography
Analysis by Electron Capture Gas Chromatography
Further Cleanup and Fractionation on Silica Gel
Alkaline Hydrolysis of Extract
Further Cleanup and Fractionation on Silica Gel
Analysis of Fractions Individually or Combined by GC/MS

Figure 1. Analytical scheme for isolation of pesticide residues from adipose tissue and liver tissue

EXPERIMENTAL

Extraction and Cleanup. Human adipose tissue and liver tissue obtained from the Dade County Medical Examiner's office were used as working samples in this investigation. It was desired to obtain tissue samples containing a relatively large variety of chlorinated hydrocarbon residues and in as wide a concentration range as possible. To provide a reasonably thorough and extensive cleanup procedure, several available methods were combined in the analytical procedure. The final scheme used in this work is shown in Figure 1. For each determination, 5 g of tissue was analyzed. Methods described in the Pesticide Analytical Manual (Food and Drug Administration, 1966) were used for initial extraction, liquid-liquid partitioning, Florisil column cleanup, and electron capture gas chromatographic analysis of the residues. An alkaline hydrolysis was performed on the extract obtained from fraction 2 of the Florisil column cleanup because of the large quantities of lipids and other tissue extractives co-eluted with the pesticide residues.

The individual extracts from fraction 1 and fraction 2 of the Florisil chromatographic step were then concentrated and subjected to further cleanup and pesticide residue fractionation by silica gel column chromatography, employing a published procedure (Kadoum, 1967). The concentrated extracts were chromatographed on 10 g of silica gel with the solvents and solvent volumes reported previously (Kadoum, 1968). The pesticide residues were thus distributed among 10 column chromatographic eluent fractions. The order of elution of the individual pesticides has been elaborated by Kadoum.

Selected chromatographic fractions containing the desired pesticide residues were then combined to form two larger fractions, the first of which contained isomers of DDT and DDE, and the second contained DDD, isomers of HCH, and the cyclodiene-type pesticides. Considerable care was used in the various solvent concentration steps to avoid loss of pesticides and permit injection of virtually the entire extract into the gas chromatograph after the final column chromatographic step. Thus, solvent volumes were reduced to 0.5 ml by Kuderna-Danish evaporative concentrators. This volume of solvent containing the pesticide residues was then transferred quantitatively to a 2-ml concentrator tube and evaporated using a stream of clean, dry nitrogen

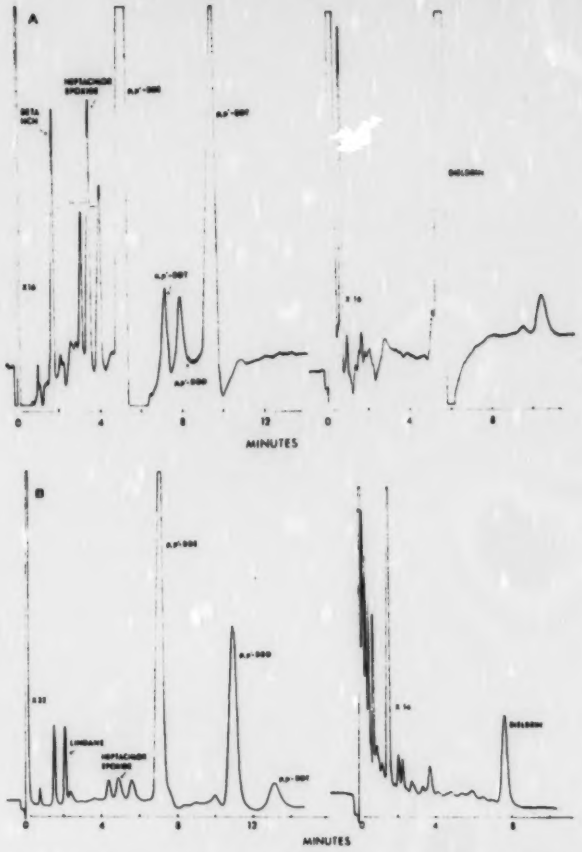

Figure 2. Electron capture gas chromatograms of pesticides isolated from human tissue

Column, mixed phase 1.5% OV-17/1.95% QF-1 on Chromosorb W (HP); column temperature 200° C; detector temperature 215° C; injector temperature 225° C

A. Upper trace. Adipose tissue extracts, fractions 1 and 2 immediately after Florisil column cleanup, nitrogen gas flow 45 ml/min, chart speed ¼ in./min

B. Lower trace. Liver tissue extracts, fractions 1 and 2 immediately after Florisil column cleanup, nitrogen gas flow 70 ml/min, chart speed ½ in./min

to a final volume of 5 μl, which was then analyzed by gas chromatography–mass spectrometry.

The silica gel column chromatographic procedure was utilized in an attempt at further cleanup of the sample extract and as a pesticide fractionation step designed to allow the analyst to choose residues or groups of residues for analysis by gas chromatography–mass spectrometry. The relatively large diameter and short length of the gas chromatography columns used in the mass spectrometric analysis resulted in somewhat low separation efficiencies for several of the pesticides found in the tissue samples, and consequently, prior separation of some of the pesticides was required.

Electron Capture Gas Chromatography Determination. The individual fractions collected from the Florisil column step were analyzed by gas-liquid chromatography using electron capture detection. Minimum sample loss occurred at this step. A Micro-Tek MT 220 gas chromatograph equipped with a ^{3}H electron capture detector was used. The conditions under which the analytical separation were carried out are given in Figure 2. Table I lists the chlorinated hydrocarbon residues found and their respective concentrations

Table I. Identity and Concentration of Pesticide Residues Found in Human Tissue Samples

A. Adipose Tissue		B. Liver Tissue	
Pesticide	Concentration (ppm)	Pesticide	Concentration (ppm)
β-HCH	0.96	Lindane	0.40[a]
Heptachlor epoxide	0.52	Heptachlor epoxide	0.07
p,p'-DDE	28.71	*p,p'*-DDE	3.56
Dieldrin	1.04	Dieldrin	0.34
p,p'-DDD	0.30	*p,p'*-DDD	2.00
o,p'-DDT	0.63	*p,p'*-DDT	0.45
p,p'-DDT	7.65		

[a] A total of 2 μg of lindane was added to the liver tissue sample prior to extraction and cleanup.

based on relative retention and peak height data obtained from reference standard pesticide chromatograms. Since no recovery studies were performed utilizing the combined extraction, liquid-liquid partitioning, Florisil column cleanup, and silica gel chromatographic fractionation methods, it should be pointed out that the quantitative data given in Table I represent approximations of the actual concentrations of residues in the tissue extract finally analyzed by gas chromatography–mass spectrometry. However, recovery data for the individual steps of the described method are well documented in the original literature references and are uniformly acceptable for the individual pesticide residues found in the human tissue samples studied.

Gas Chromatography–Mass Spectrometry. The mass spectrometric analyses were performed with a double focusing, medium resolution mass spectrometer (Model 270, Perkin-Elmer Corp., Norwalk, Conn.) coupled through a Watson-Biemann type of molecular separator with a gas chromatographic system. The coiled glass gas chromatographic column was 4 ft long by ¼ in. o.d., and was packed with 3% OV-210 on 80 100 mesh Chromosorb W (HP) obtained from the Pierce Chemical Co., Rockford, Ill. Programmed temperature analyses were made with the oven initially at 150° C for 1 min, followed by a 5°/C min increase to 200° C; finally, the oven was held isothermally at 200° C. The molecular separator and gas inlet temperatures were maintained at 150 and 210° C, respectively. All mass spectra were recorded at 80 e.v. electron energy with 2300 V accelerating voltage; the filament emission current was 100 μa. Chromatograms were recorded from the total ion-current monitor located between the electrostatic and magnetic analyzer sectors. Helium carrier gas flow rate was 10 ml per min. Injector temperature was 165° C. Mass spectra were scanned magnetically over the range *m/e* 5 to 500 in 6 sec.

In the initial phases of this work considerable decomposition of pesticides injected into the gas chromatographic unit occurred in the stainless steel restricter line, transfer line, and fritted glass molecular separator of the manifold system. This degradation was manifested as a diminished intensity molecular ion peak for several pesticidal compounds examined, including *p,p'*-DDT and lindane. In some cases, pesticide identity was confused, particularly at concentrations below 1 μg. The following instrumental modification and silanization treatment greatly alleviated this problem. The stainless steel transfer line, restricter line, and Kovar glass-to-metal seal portion of the molecular separator were removed and replaced with glass capillary tubing to minimize contact of eluted pesticides with hot metal surfaces. The entire glass separator assembly was then washed with hot nitric acid, water, acetone, benzene, and hexane, silanized with N,O'-bis-trimethyl silyl acetamide (Pierce Chemical Co., Rockford, Ill.), and reinstalled. In addition, polar chemisorption was further deactivated by silanization *in situ* of the gas chromatographic column, fritted glass molecular separator, and transfer lines by using a published procedure (MacLeod and Nagy, 1968).

Heptachlor epoxide (1,4,5,6,7,8,8-heptachloro-2,3-epoxy-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene) was determined by a recently developed procedure for enhancing mass spectral data by means of a time averaging computer (Biros, 1970). Repetitive rapid scanning and spectrum accumulation in the region of the parent molecular ion for heptachlor epoxide produced a considerably enhanced isotopic distribution pattern characteristic of a molecule containing seven chlorine atoms and having a molecular weight of 386.

RESULTS AND DISCUSSION

Mumma and Kantner (1966) reported that 0.1 ppm of halogenated pesticides in alfalfa and wheat samples gave sufficient residues for analysis by mass spectrometry. These workers utilized a gas chromatographic trapping technique in conjunction with mass spectrometric analysis by a direct introduction probe. Residues as small as 0.1 μg were determined in this fashion. Because of the relatively large diameter of the gas chromatographic columns utilized in this work and the probability that up to 60% of the sample is lost by using a fritted glass tube molecular separator (Grayson and Wolf, 1967), it was anticipated that the minimum sample required for analysis would be of the order of 0.2 to 0.3 μg, corresponding to 0.04 to 0.06 ppm of the pesticide residue based on the quantity of tissue analyzed. Examination of the electron capture gas chromatogram (Figure 2) of the adipose tissue sample reveals that seven pesticide residues were present in concentrations ranging from 0.30 to 28.7 ppm (Table I). By relative retention time data, these were determined to be the β-isomer of HCH; heptachlor epoxide; 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE), 1,2,3,4,10,10 - hexachloro - 6,7 - epoxy - 1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo,exo*-5,8-dimethanonaphthalene (dieldrin), 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethane (*p,p'*-DDD), *o,p'*-DDT, and *p,p'*-DDT. The pesticide residues were conveniently separated by silica gel chromatography into two fractions, each of which contained pesticides eluted with sufficient resolution for proper identification. The total ion current traces of the adipose tissue extracts are shown in Figure 3. The mass spectra of authentic reference compounds were compared with those of the components of the extract to identify the following pesticides. (Discussions of fragmentation modes, mass spectral peak intensities, and identities of pesticides under electron impact-induced ioniza-

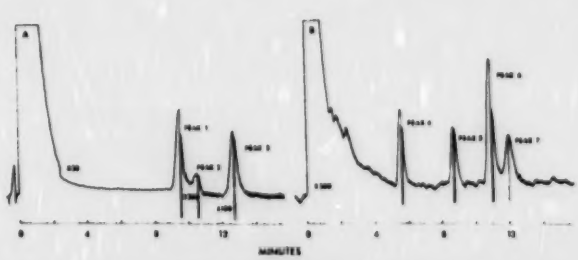

Figure 3. Total ion current traces of extracts containing pesticide residues isolated from adipose tissue (A) fraction 1; (B) fraction 2 (see text)

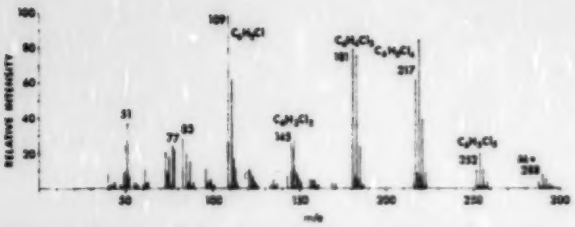

Figure 4. Mass spectrum of the β-isomer of 1,2,3,4,5,6-hexachlorocyclohexane isolated from adipose tissue

tion have been reported by Damico *et al.*, 1968; Jörg *et al.*, 1966; and Kantner and Mumma, 1966).

Peak 1. *p,p'*-DDE. *m/e* 246 (100%), 318 (83%), 316 (molecular ion, 66%), 248 (58%), 320 (41%), 176 (41%), 210 (16%).

Peak 2. *o,p'*-DDT. *m/e* 235 (100%), 237 (70%), 165 (40%), 75 (20%), 199 (18%), 246 (18%), 352 (molecular ion, 3%).

Peak 3. *p,p'*-DDT. *m/e* 235 (100%), 237 (72%), 165 (48%), 75 (22%), 50 (18%), 51 (9%), 352 (molecular ion, 2%).

Peak 4. β-HCH. *m/e* 109 (100%), 219 (85%), 181 (78%), 183 (80%), 111 (62%), 193 (62%), 288 (molecular ion, 4%).

Peak 5. Heptachlor epoxide. *m/e* 81 (100%), 353 (84%), 355 (76%), 351 (48%), 357 (35%), 237 (33%), 386 (molecular ion, 8%).

Peak 6. Dieldrin. *m/e* 79 (100%), 108 (19%), 263 (18%), 277 (19%), 279 (16%), 345 (7%), 378 (molecular ion, 3%).

Peak 7. *p,p'*-DDD. *m/e* 235 (100%), 237 (66%), 165 (58%), 75 (21%), 82 (16%), 88 (16%), 318 (molecular ion, 5%).

As expected, the mass spectrum of β-HCH (Figure 4) at low resolution and at the concentrations encountered in the analysis was indistinguishable from spectra of reference samples of the three remaining isomers (α-, γ-, and δ-HCH) occasionally found as residues in human tissue. Consequently, identification of this pesticidal compound as β-HCH must be corroborated by gas chromatographic retention time data. A similar difficulty arose with *o,p'*-DDT and *p,p'*-DDT. At high concentrations and under controlled conditions, these configurational and structural isomers can be differentiated by ion intensity ratios of characteristic ion fragments (Damico, 1969). However, at the lower concentrations at which they are encountered as residues and under the conditions of the analysis, the mass spectral properties of *o,p'*-DDT and *p,p'*-DDT are too similar to allow identification on this basis alone, and their identification requires gas chromatographic retention time data in addition to mass spectral fragmentation patterns, peak intensities, and molecular weights. Adequate and definitively characteristic mass spectral data were, however, obtained for the remaining pesticide residues isolated from the adipose tissue sample.

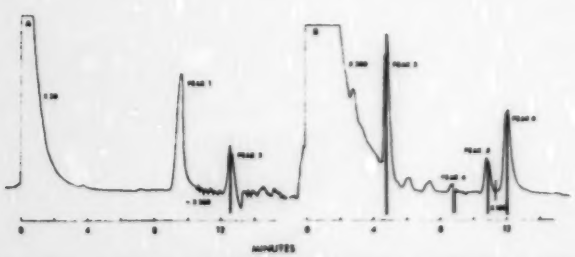

Figure 5. Total ion current traces of extracts containing pesticide residues isolated from liver tissue (A) fraction 1; (B) fraction 2 (see text)

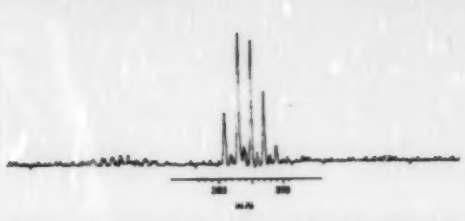

Figure 6. Computer time averaged spectrum (10 scans) of the molecular ion region of heptachlor epoxide, *m/e* 380 to 400, isolated from liver tissue

The total ion current traces of the residue extracts of the liver tissue sample are presented in Figure 5. Five pesticides were isolated from the liver tissue, as shown in Table I. Lindane at 0.4 ppm was added to the tissue sample prior to extraction. Pesticides were identified on the basis of the following mass spectral data.

Peak 1. *p,p'*-DDE. *m/e* 246 (100%), 318 (80%), 316 (molecular ion, 63%), 248 (59%), 320 (39%), 176 (44%), 210 (18%).

Peak 2. *p,p'*-DDT. *m/e* 235 (100%), 237 (69%), 165 (51%), 75 (25%), 50 (20%), 51 (12%), 352 (molecular ion, 3%).

Peak 3. Lindane. *m/e* 109 (100%), 111 (79%), 181 (96%), 183 (96%), 219 (93%), 217 (80%), 51 (46%), 288 (molecular ion, 3%).

Peak 4. Heptachlor epoxide. Molecular ion isotope group at *m/e* 386, 388, 390, 392, 394 (Figure 6).

Peak 5. Dieldrin. *m/e* 79 (100%), 108 (16%), 263 (15%), 277 (18%), 279 (16%), 345 (8%), 378 (molecular ion, 3%).

Peak 6. *p,p'*-DDD. *m/e* 235 (100%), 237 (69%), 165 (52%), 75 (18%), 82 (15%), 88 (15%), 318 (molecular ion, 6%).

Relative ion intensity data of the mass spectra of reference samples of the four isomers of HCH were compared with the mass spectrum of Peak 1.

Data again showed too wide a discrepancy to allow confirmation of lindane on this basis alone. Gas chromatographic retention time data, however, permitted definitive identification. Heptachlor epoxide was identified by accumulating 10 scans of the mass spectrum of Peak 4 in the region of *m/e* 380 to 400. Figure 6 illustrates the results of the computer scans plotted in analog form on an X-Y recorder. The characteristic isotopic distribution pattern for seven chlorine atoms is clearly recognizable. These data, along with the molecular weight obtained from this group of mass spectral peaks (386 amu), and gas chromatographic retention time data, identified Peak 4 as heptachlor epoxide. The remaining pesticides were directly identified by comparing mass spectral data with those of reference standard materials and literature mass spectra.

CONCLUSIONS

The combined gas chromatography–mass spectrometry technique has been successfully applied to the identification and confirmation of pesticide residues isolated from human adipose and liver tissue samples. A general extensive extraction and cleanup technique adapted from existing methods was used to isolate and purify residues. Pesticides were separated by a silica gel chromatographic step into two fractions with sufficient resolution on the gas chromatographic column for identification of seven pesticide residues from the adipose tissue sample and six pesticide residues from the liver tissue sample. These include: β- and γ-HCH, heptachlor epoxide, dieldrin, *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT, and *p,p'*-DDT.

Instrumental sensitivity was sufficient to identify residues at concentrations of 0.30 ppm. Recently developed computer enhancement techniques permitted identification of heptachlor epoxide residues at a level of 0.073 ppm. Definitive confirmation of pesticide residues was obtained by comparison of parent and fragment ion intensities and mass numbers of eluted residues and reference pesticides. Some difficulties were encountered in the absolute confirmation of configurational and structural isomers where mass spectral peak intensities and mass values of characteristic fragment ions were too similar under the conditions of the analysis. In these instances, however, identification was made readily on the basis of gas chromatographic retention times. The estimated lower limit of detectability of pesticide residues by the analytical scheme and instrument configuration reported is approximately 0.05 to 0.1 ppm. This can be improved considerably by instrumental modifications utilizing either glass capillary gas chromatographic columns with direct entry to the ion source or possibly other types of molecular separators such as the membrane type, which provide somewhat greater sample recoveries. In addition, the inclusion of an electron capture detector and splitter arrangement in tandem with the molecular separator would allow simultaneous gas chromatographic recording of pesticide effluents with minimum sacrifice of sample. Thus mass spectrometric identification and electron capture detection and quantitation of pesticide residues from a single gas chromatographic injection would be possible. Work is currently in progress on these and related areas of pesticide residue analysis and confirmation by GC-MS.

LITERATURE CITED

Amy, J. W., Chait, E. M., Baitinger, W. E., McLafferty, F. W., *Anal. Chem.* **37**, 1265 (1965).
Beynon, J. H., "Mass Spectrometry and Its Applications to Organic Chemistry," p. 298, Elsevier Publishing Co., New York, 1960.
Biros, F. J., *Anal. Chem.* **42**, 537 (1970).
Damico, J. N., Food and Drug Administration, personal communication, 1969.
Damico, J. N., Barron, R. P., Ruth, J. M., *Org. Mass Spectrom.* **1**, 331 (1968).
Damico, J. N., Wong, N. P., Sphon, J. A., *Anal. Chem.* **39**, 1045 (1967).
Elgar, K. E., *J. Ass. Offic. Anal. Chem.* **50**, 1067 (1967).
Food and Drug Administration, Pesticide Analytical Manual, Vol. I, Sec. 211, U.S. Dept. of Health, Education, and Welfare, Washington, D.C., 1966.
Grayson, M. A., Wolf, C. J., *Anal. Chem.* **39**, 1438 (1967).
Jörg, J., Houriet, R., Spiteller, G., *Monatsh. Chem.* **97**, 1064 (1966).
Kadoum, A. M., *Bull. Environ. Contam. Toxicol.* **2**, 264 (1967).
Kadoum, A. M., *Bull. Environ. Contam. Toxicol.* **3**, 65 (1968).
Kantner, T. R., Mumma, R. O., in "Residue Reviews," Vol. 16, p. 138, F. A. Gunther, Ed., Springer-Verlag, New York, 1966.
MacLeod, W. D., Nagy, B., *Anal. Chem.* **40**, 841 (1968).
McFadden, W. H., *Separ. Sci.* **1**, 723 (1966).
Muller, D. O., *Anal. Chem.* **35**, 2033 (1963).
Mumma, R. O., Kantner, T. R., *J. Econ. Entomol.* **59**, 491 (1966).
Robinson, J., Richardson, A., Elgar, K. E., Abstracts, 152nd National Meeting ACS, New York, Sept. 1966, Div. of Agricultural and Food Chemistry, No. A-75.
Schechter, M. S., *Pestic. Monit. J.* **2**, 1 (1968).
Watson, J. T., Biemann, K., *Anal. Chem.* **37**, 844 (1965).

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SHORT COMMUNICATION

The direct determination of cadmium in biological samples by selective volatilization and graphite tube reservoir atomic absorption spectrometry

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(Received 2nd August 1973)

The biological influences of cadmium have been reviewed by Underwood[1] and Friberg *et al.*[2]. From an environmental point of view, the metal is one of the most dangerous of the atmospheric pollutants[3]. It has been shown that an estimated 100 human deaths have resulted from environmental cadmium poisoning[4]. In man, cardiovascular disease[5] and arterial hypertension[6] have been associated with inhaled doses of the metal toxicant. When administered to rats, the pollutant produces hypertension[7] and increased mortality[8].

As with mercury, very little is known about the fate and distribution of cadmium in the environment, but as greater quantities of the metal are refined, more of it becomes available to interact with man. Before the biological effects of cadmium can be completely understood, analytical methods which may be used to determine lengthy exposure of humans at low concentrations must be available. The method must be sensitive and relatively simple, in order to determine cadmium in large numbers of biological and environmental samples.

Several analytical methods for determining trace quantities of the metal have been reported[2]. Atomic absorption spectrometry (a.a.s.) is most widely used[9]. Extraction procedures for use with a.a.s. by conventional flame techniques for determining trace cadmium have been published[10,11]; because of the low concentrations found in biological matrices, direct determination is not feasible. In order to monitor human exposure to cadmium poisoning, it is important to have a method available in which the metal may be determined directly from biological samples thus eliminating the possibility for loss encountered in sample digestion and workup. Pulido *et al.*[10] achieved this by using flame a.a.s. interfaced with a vycor absorption cell, whereas Hauser *et al.*[11] developed a reagent-free method for the determination of both cadmium and lead in whole blood, employing a flame-purged tantalum sampling boat. The development of atom reservoirs for use with a.a.s., such as the graphite tube furnace (HGA) and the carbon rod atomizers, have increased the sensitivity of the technique by several orders of magnitude. The applications of these methods have been reviewed by Kirkbright[12]. A direct HGA method has been reported for the determination of low quantities of chromium in human urine samples[13]. It was the purpose of this investigation to adapt this system for the direct determination of cadmium in urine and serum.

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Experimental

Apparatus. A Perkin Elmer model 303 atomic absorption spectrophotometer equipped with a Graphicorder-10 (Dynatronic Instruments), a deuterium arc background corrector and a Perkin Elmer heated graphite atomizer (HGA-2000) were used. The HGA-2000 provides variable temperature programs and controls which eliminate the need for voltage temperature conversion tables and electronic modifications, and allows selective volatilization analyses to be performed for all samples[14]. The temperatures are also reported in voltages which are measured directly across the atomizer terminals. These corrected values are used throughout the paper because of the discrepancy between applied voltages and the actual temperatures achieved in the atomizer. The actual temperature obtained in the graphite tube is a function of the geometry and age of the tube[14].

The instrument was operated according to the standard conditions for cadmium recommended by the manufacturer. An inert atmosphere was maintained by purging the graphite tube with nitrogen at 1.5 l min $^{-1}$. Eppendorf microliter pipettes with disposable plastic tips were used for injections of the samples into the atomizer.

Reagents. Working standard solutions were prepared daily by diluting a 1000 p.p.m. cadmium reference solution (Fischer Scientific Co.) with doubly distilled deionized water which showed no cadmium absorption when analyzed by the HGA technique.

Urine samples were collected in polyethylene containers which had been pre-rinsed with quartz-distilled nitric acid. The samples were acidified and stored at 4 C. Serum samples were obtained from blood that had been collected in plastic syringes (which were checked for possible cadmium contamination) and allowed to coagulate in polyethylene centrifuge tubes.

Procedure. Aliquots (20 μl) of either urine or 20% solutions of serum in water were introduced into the graphite tube with disposable micropipettes. (Calibration curves were obtained by injecting cadmium working standard solutions and by fortifying cadmium-free urine or serum with identical amounts of cadmium.)

The HGA power supply was programmed as follows: drying, 30 s at 150 C (0.6 V); charring, 50 s at 450 C (1.2 V); atomizing, 8 s at 1300 C (2.0 V). The temperatures were read directly from the meter on the power supply unit, and the corresponding voltages were measured across the atomizer terminals by means of a voltmeter.

After each analysis, the graphite tube was heated to maximum temperature for 10 s; this was necessary because salts such as sodium chloride were not completely volatilized during the 8 s atomization step at 1300 C. No apparent aging of the graphite tube was found with up to 100 sample injections. The temperatures and times applied during the various steps are critical because many samples contain large amounts of dissolved salts which, if not removed by selective volatilization, would produce light scattering and higher apparent cadmium signals.

Samples were analyzed by direct injection into the graphite atomizer and by the chelation extraction method described by Lehnert *et al.*[15,16].

The relative standard deviation (s_r) for peak signals calculated from ten injections of 20 μl of each urine, serum and aqueous standard solutions con-

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taining 5 p.p.b. ($1 \cdot 10^{-10}$ g) of cadmium were 4.1%, 5.8%, and 4.9%, respectively. This reproducibility is in close agreement with that cited in the literature[14,17,18].

The concentration of cadmium in urine or serum needed to produce a 1% absorption signal under the above conditions was found to be 0.25 $\mu g\ l^{-1}$ (0.25 p.p.b.).

Results and discussion

The initial application of the method for the measurement of cadmium in biological materials resulted in spectral and chemical interferences which were due to the formation of smoke produced from charring of the organic matter and inorganic salts such as sodium chloride in the matrix. This resulted in non-specific absorption and led to high cadmium readings. Elimination of these interferences was accomplished by using a deuterium arc background corrector. Figure 1 shows tracings from injections of 20-μl samples of urine fortified with 3 p.p.b. of cadmium both with and without the deuterium arc background corrector in the system. At 1700 C, cadmium was covolatilized with the matrix salts (A, Fig. 1). At higher temperatures light scattering due to salt volatilization resulted to an extent that precluded compensation, and the cadmium peak was only partly resolved. However, at 1300 C, the cadmium peak was clearly differentiated from the peak produced from light scattering even without the deuterium arc corrector (B, Fig. 1). At this temperature with the deuterium arc background corrector, a cadmium tracing essentially free of interferences was obtained (D, Fig. 1). Evidence

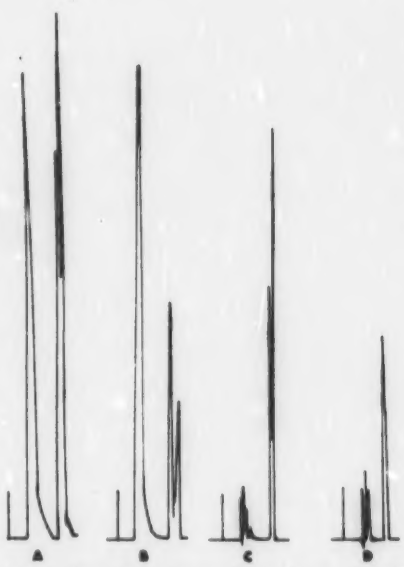

Fig. 1. Recorder tracings obtained from the HGA 2000 as a function of temperature and deuterium arc background correction. (A) 1700 C with deuterium background corrector off; (B) 1300 C with deuterium background corrector off; (C) 1700 C with deuterium background corrector on; (D) 1300 C with deuterium background corrector on.

for the authenticity of the cadmium signal was obtained by fortifying serum and urine samples with 100 p.p.b. of cadmium. The samples were then extracted with an excess of ammonium pyrrolidine dithiocarbamate (APDC) and methyl isobutyl ketone (MIBK) until the cadmium peak completely disappeared. Possible salt interferences from urine samples were also checked by adding to the samples 5 p.p.b. of cadmium. The samples were injected into the HGA, and the peak height recorded. The same samples were diluted to 2, 4, 8 and 16 times the original volumes, respectively, and the cadmium content was readjusted to its initial concentration of 5 p.p.b. The samples again were injected into the HGA, and the signal recorded. The cadmium peak heights for the diluted urine samples were the same as the cadmium peaks obtained in the original undiluted urine samples. These data illustrate that the response for cadmium is not affected by salt content in urine under the operating conditions described above. Pulido *et al.*[10] and Hauser *et al.*[11] have discussed the use of deuterium arc background correctors for the determination of cadmium in biological samples, using a.a.s. in conjunction with other sampling techniques. Their data is in close agreement with the data reported here.

Levels of cadmium found in the urine of nonexposed individuals are shown in Table I. The samples were analyzed by the proposed direct injection technique and also by an extraction method[15]. It can be seen that there is close agreement between the results obtained.

TABLE I

COMPARISON OF URINE CADMIUM LEVELS BY PROPOSED METHOD AND BY EXTRACTION METHOD

(Results in $\mu g\ l^{-1}$)

Sample	1	2	3	4	5	6	7	8	9
Proposed method	1.8	1.0	1.6	0.6	1.1	1.7	2.7	2.4	2.5
Extraction	1.8	1.0	1.9	0.9	1.1	1.6	3.0	2.0	2.5

TABLE II

DETERMINATION OF TRACE QUANTITIES OF CADMIUM IN URINE AND SERUM

Sample	*No. of samples*	*Range* $\mu g\ l^{-1}$	*Mean and std deviation*
Urine	20	0.4–3.7	1.5 ± 0.7
Serum	5	7.5–9.5	8.8 ± 0.7

Table II illustrates applications to serum and urine. The levels of cadmium found in nonexposed urine samples are in agreement with those reported in the literature[11–15, 16, 19, 20]. The values reported for serum are intermediate between the levels reported by Lehnert *et al.*[15] and Pulido *et al.*[10].

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The data reported here for cadmium levels in urine and serum indicate a high potential for the direct application of a.a.s. in conjunction with the HGA and a deuterium arc background corrector to biological samples. Its sensitivity, simplicity and speed make this procedure by far superior to other previously reported analytical techniques for the determination of this element.

REFERENCES

1 J. F. Underwood, *Trace Elements in Human and Animal Nutrition*, Academic Press, New York, 3rd ed., 1971.
2 L. Friberg, M. Piscator and G. Nordberg, *Cadmium in the Environment*, CRC Press, Cleveland, Ohio, 1971, p. 16.
3 C. M. Christian II and J. W. Robinson, *Anal. Chim. Acta*, 56 (1971) 466.
4 N. Yamagata and I. Shigematsu, *Bull. Inst. Pub. Health, Tokyo*, 19 (1970) 1.
5 R. J. Carroll, *J. Amer. Med. Ass.*, 198 (1966) 267.
6 H. A. Schroeder, *Arch. Environ. Health*, 21 (1970) 798.
7 H. A. Schroeder and W. H. Vinton Jr., *Amer. J. Physiol.*, 203 (1962) 515.
8 H. A. Schroeder, W. H. Vinton Jr. and J. J. Balassa, *J. Nutr.*, 80 (1963) 48.
9 W. Slavin, *Atomic Absorption Spectroscopy*, Interscience, New York, 1968, p. 86, 87.
10 P. Pulido, K. Irena and B. L. Valla, *Anal. Biochem.*, 14 (1966) 393.
11 T. R. Hauser, T. A. Hinners and J. L. Kent, *Anal. Chem.*, 44 (1972) 1819.
12 G. F. Kirkbright, *Analyst*, 96 (1971) 609, 1146.
13 R. T. Ross, J. G. Gonzalez and D. A. Segar, *Anal. Chim. Acta*, 63 (1973) 205.
14 D. A. Segar and J. G. Gonzalez, *At. Absorption Newslett.*, 10 (1971) 94.
15 G. Lehnert, K. H. Schaller and T. Haas, *Z. Klin. Chem.*, 6 (1968) 174.
16 G. Lehnert, G. Klavis, K. H. Schaller and T. Haas, *Brit. J. Ind. Med.*, 26 (1969) 1156.
17 E. Norval and L. R. P. Butler, *Anal. Chim. Acta*, 58 (1972) 47.
18 M. D. Amos, D. A. Bennett, K. G. Brodie, P. W. Y. Lung and J. P. Matousek, *Anal. Chem.*, 43 (1971) 211.
20 H. M. Perry, *Trace Substances in Environmental Health – II*, University of Missouri, 1969, p. 101.
21 D. I. Hammer, J. F. Finklen, J. P. Creason, S. M. Sandifer, J. F. Keil, L. F. Priester and J. F. Stara, *Trace Substances in Environmental Health – II*, University of Missouri, 1969, p. 269.

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The Determination of Methyl Mercury in Urine

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Urinary and fecal (both bile and intestinal mucosa) routes of excretion are the most important for the elimination of mercury. Elimination via the sweat and salivary glands although a minor excretory pathway may be of importance diagnostically (INTERNATIONAL COMMITTEE 1969, SUZUKI et al. 1966, JOSELOW et al. 1968). For monitoring levels of mercury in man, urinary and fecal routes of excretion are the most important. However, from a practical point of view, the urinary excretion of mercury is of special interest, and if urine analyses are to be used to monitor exposure, an analytical method specific for alkyl mercuries is desirable (INTERNATIONAL COMMITTEE 1969).

Several methods are reported in the literature for the rapid determination of total mercury in urine (MAGOS and CERNIK 1969, LINDSTEDT and SKARE 1970, LINDSTEDT and SKARE 1971). These methods are both sensitive and specific but do not have the versatility to differentiate between inorganic and organic forms of mercury.

To date, the most popular and widely used method for determining alkyl mercuries in tissue samples is the WESTÖÖ procedure (WESTÖÖ 1966, WESTÖÖ 1967, WESTÖÖ 1968). This method involves a benzene extraction of the acidified tissue homogenate, a cleanup of the benzene extract with an aqueous solution of cysteine acetate and a final extraction of the alkyl mercury salt from the cysteine solution with benzene. The benzene extract is analyzed using gas liquid chromatography (GLC) equipped with an electron capture (EC) detector system. This procedure has been used extensively by Kamps and McMahon for determining alkyl mercuries in fish tissues (KAMPS and McMAHON 1972).

This paper describes a modification of the above procedure for the determination of methyl mercury in urine employing a cysteine acetate cleanup procedure and analysis using the GLC-EC technique.

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EXPERIMENTAL

Reagents

Benzene (pesticide quality), the purity of benzene is such that an injection of 10 µl from a 100 ml aliquot concentrated to 10 ml shows no response other than the benzene peak.

Hydrochloric acid (Ultrex, J.T. Baker Chemical Co.), Ultra high purity concentrated hydrochloric acid was extracted in a separatory funnel several times with Nanograde benzene until injections of the organic layer did not exhibit any GLC peaks after the solvent front.

Water, glass distilled, doubly deionized, is extracted in a separatory funnel several times with benzene.

Cysteine acetate solution, (K & K Laboratories Inc., Plainview, N.Y.), was prepared by the procedure described by West88 (WESTÖÖ 1966, WESTÖÖ 1967, WESTÖÖ 1968).

Methyl Mercuric Chloride, MMC (K & K Laboratories Inc., Plainview, N.Y.), 98% purity.

Apparatus

Gas chromatograph-Microtek, Model MT 220, equipped with an EC detector and U-shaped glass GC columns 1/4" x 6' packed with 5% HIEFF-2AP on Chromosorb W 80/100 mesh.

Temperatures: Inlet 200°C; column 170°C; transfer lines and outlet block 200°C. The polarizing voltage is set to produce a maximum response for an injection of 1.0 ng of methyl mercuric chloride.

Centrifuge, Model CL, International Clinical Centrifuge.

Procedure

All glassware is washed according to standard laboratory procedure, and then rinsed with ammonium hydroxide, deionized-distilled water, ethanol and benzene.

A 15 ml urine sample is pipetted into a 50 ml stoppered centrifuge tube and acidified to pH 1 with concentrated hydrochloric acid. To the mixture is added 15 ml of benzene, and the tube is shaken vigorously for 5 minutes. The emulsion is broken by centrifugation, and the organic layer is removed by means of a disposable pipette. The urine is extracted again as described above with another 15 ml portion of benzene and centrifuged. The organic layers are combined, and a 25 ml aliquot is removed and extracted with 5 ml of the cysteine acetate solution. The mixture is shaken vigorously and the layers separated. To a 10 ml stoppered centrifuge tube is added a 4 ml aliquot of the aqueous cysteine solution which is then acidified to a pH 1 with hydrochloric acid and extracted with two 2.5 ml portions of benzene. The benzene layers are combined, dried over anhydrous sodium sulfate and stored in a glass stoppered test tube until GLC analysis can be performed.

A calibration curve is prepared for each set of samples from data obtained by substitution of water for urine and using identical volumes of reagents and benzene for the extraction.

Recovery data were obtained by fortifying samples of urine with MMC.

RESULTS AND DISCUSSION

Recovery data obtained for urine samples which had been fortified with MMC are shown in Table 1.

TABLE 1

Recovery Data of Methyl Mercuric Chloride from Urine

No.of Samples	Level (ppb)	Recovery %
4	36	103.7
2	18	104.0
2	9	99.0

Analysis of the benzene extract afforded quantitative recovery of MMC. Direct benzene extraction of unacidified urine samples which had been fortified with MMC resulted in no recovery of MMC. The failure to detect MMC from unacidified urine samples indicates

that the binding between MMC and the constituents of urine take place rapidly and completely (SUMINO 1968a, SUMINO 1968b).

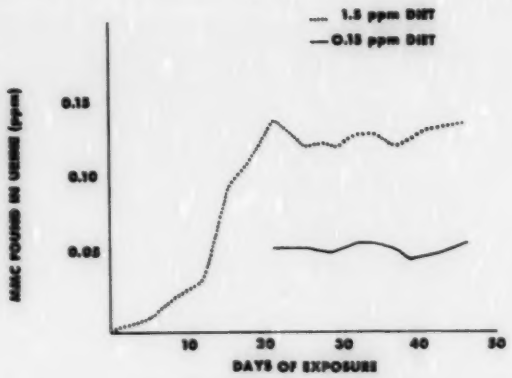
FIGURE 1

Figure 1 shows chromatograms of a benzene extract from acidified urine samples before and after cysteine cleanup. The chromatograms illustrate the need for the cleanup procedure since interfering compounds elute from the GLC column in the area where MMC appears.

The EC detector system is linear in the range of 0 to 2.5 ng of MMC. The detector is sensitive to 5 picograms of MMC with a signal to noise ratio of 3/1.

In order to examine the efficacy of this method, two groups of rats were fed a diet containing 1.5 and 0.15 ppm of MMC for one and two months respectively, and the amount of MMC excreted in the urine was monitored. A plot was made of the amount of MMC found in the urine as a function of the number of days of exposure, Figure 2. The group which was fed 1.5 ppm showed

very little MMC excreted up to twelve days. After this time, the amount excreted increased rapidly up to approximately twenty-one days after which a leveling off effect was observed. No MMC could be found in the urine of rats which were fed 0.15 ppm until the 21st day of exposure. After this time, the amount excreted remained approximately constant for 30 days.



EXCRETION OF MMC IN THE URINE OF RATS

FIGURE 2

The data indicate that MMC is excreted in the urine after some build up of the compound in various tissue sites has taken place. This is illustrated by the fact that no MMC was found in the urine of rats fed a diet containing 0.15 ppm until after 21 days, whereas urinary excretion of MMC in the group of rats on the 1.5 ppm MMC diet began much earlier in the experiment. No attempt was made to quantitate the amount of MMC which could have been converted to inorganic mercury and excreted as such (GAGE 1964).

In summary, MMC is excreted in the urine of animals exposed to this material through dietary intake. Since urinary excretion is of special interest for monitoring exposure to toxic substances, the data produced in this investigation suggests the possibility of monitoring levels of MMC in the general population employing the described procedure. The method is capable of detecting ppb of MMC and combines high recoveries and rapid analysis time in a single procedure. Since binding of MMC to urine constituents is fast and complete, acidification of the urine to pH 1-2 is absolutely necessary prior to benzene extraction. The cleanup procedure using cysteine acetate solution is also essential for the elimination of interfering GLC peaks. This procedure has been used previously for determining MMC from blood and tissue, and the cleanup step was shown to be unnecessary for these samples (ROSS and GONZALEZ, 1971).

REFERENCES

GAGE, J.C., Brit. J. Industr. Med., 21, 197(1964).
INTERNATIONAL COMMITTEE, Report of an, Arch. Environ. Health, 19, 891(1969).
Joselow, M.M., R. RUIZ, and L.J. GOLDWATER, Arch. Environ. Health, 17, 35(1968).
KAMPS, L.R. and B. McMAHON, J.A.O.A.C., 55, 590(1972).
LINDSTEDT, G. and I. SKARE, Analyst, 95, 264(1970).
LINDSTEDT, G. and I. SKARE, Analyst, 96, 223(1971).
MAGOS, L. and A.A. CERNIK, Brit. J. Industr. Med., 26, 144(1969).
ROSS, R.T. and J.G. GONZALEZ, 162nd National Meeting of the American Chemical Society, Washington, D.C., Fall, 1971, Pesticide Division, Paper No. 070.
SUMINO, K., Kobe J. Med. Sci., 14, 115(1968a).
SUMINO, K., Kobe J. Med. Sci., 14, 131(1968b).
SUZUKI, T., T. MIYAMA and H. KATSUNUMA, Industrial Health, 4, 69(1967).
WESTÖÖ, G., Acta Chemica Scandinavica, 20, 2131(1966).
WESTÖÖ, G., Acta Chemica Scandinavica, 21, 1790(1967).
WESTÖÖ, G., Acta Chemica Scandinavica, 22, 2277(1968).

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Enhancement of Mass Spectral Data by Means of a Time Averaging Computer

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ALTHOUGH MASS SPECTROMETRY is in general one of the most sensitive spectroscopic tools available to the analyst, frequently providing useful data on 1 μg or less of material, in many cases it is desirable to attempt confirmation of gas chromatographic effluents of known structure by this technique at concentration levels of the nanogram range or less. A potentially important application in which such enhanced sensitivity would be particularly useful is pesticide residue analysis since residues at picogram and nanogram level are often encountered. Recent reports have focused attention on the need for confirming pesticide residues, particularly those isolated from environmental samples (*1*, *2*) and for chemical identity in ultramicroanalysis in general (*3*).

In the examination of small quantities of materials such as pesticide residues by combined gas chromatography–mass spectrometry, several factors which can influence the quality of the recorded mass spectra data must be considered (*4*). The mass spectrum of compounds emerging from a gas chromatographic column will be distorted if a change in concentration occurs during the scanning period. To minimize this distortion, the mass spectrum is scanned rapidly at the top of the gas chromatographic peak. Rapid scanning, however, can adversely affect the integrity of the mass spectrum, since the statistical fluctuations associated with the recording of small ion currents at fast rates can cause serious errors in the recorded magnitude of these currents. As a consequence, the spectrum becomes less accurate and less reproducible as the scan rate is increased and/or the sample size is decreased. Quantitative estimates of the precision limitations associated with the measurement of low ion currents have been made previously (*5–7*).

Several mass spectrometric techniques which have found utility in minimizing recorded errors caused by the effects of concentration changes in the ion source and statistical fluctuations in the measurements of small ion currents have been described. These include photographic plate detection (*8*, *9*), ratio recording of mass spectra (*4*), mass fragmentography (*10*, *11*), and pulse height analysis (*12*). Although the value of time averaging of mass spectral data for the enhancement of sensitivity and ion peak intensities has been recognized (*4*, *5*), no report is available regarding its utility in specific analytical applications. The present communication describes the interfacing of a small laboratory time averaging computer with a combination gas chromatograph-mass spectrometer, allowing repetitive scanning and averaging of mass spectra peaks, subtraction of background peaks, normalization, and printout in plotted form, and the applications of this system for the confirmation of pesticide residues of known structure.

EXPERIMENTAL

Materials. Standard solutions of pesticides were prepared in hexane in concentrations ranging from 1 ng/μl to 50 ng/μl. The chlorinated pesticides used in this work were analytical grade materials (purity at least 99% as determined by electron capture gas chromatography). 1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene (dieldrin) was obtained from Shell Chemical Co., New York, N. Y. 1,1-Bis(*p*-chlorophenyl)-2,2,2-trichloroethane (*p,p'*-DDT) and *p,p'*-DDE were obtained from the Aldrich Chemical Co., Cedar Knolls, N. J.

Instrumentation. Mass spectrometric analyses were performed with a double focusing, low resolution mass spectrometer (Model 270, Perkin-Elmer Corp., Norwalk, Conn.) coupled through a Watson-Biemann type of molecular separator with a gas chromatographic system. The coiled glass gas chromatographic column was 4 feet × 1/8 inch o.d., and was packed with 3% OV-210 on 80/100 mesh Chromosorb W (HP) obtained from the Pierce Chemical Co., Rockford, Illinois. Isothermal analyses were made at column temperatures which gave retention times of 10–15 minutes for each of the pesticides (*ca.* 180 °C). The molecular separator and gas inlet were held at 200 °C. The ion source and target temperatures were maintained at 150 °C and 210 °C, respectively. All mass spectra were recorded at 80 eV electron energy with 2000 volts accelerating voltage and the filament emission current was 100 μa. The modifications to the manifolding system of the gas chromatograph–mass spectrometer have been described elsewhere (*13*).

In addition, further deactivation of polar chemisorption by silanization of the gas chromatographic column, fritted glass molecular separator, and transfer lines were performed on the intact system by a published procedure (*14*).

The mass spectrometer was focused on certain *m/e* values by manual adjustment of the start and stop scan controls. This technique, which requires some time and patience, was normally carried out before injection of the sample. The mass spectrometer scan was started at the upslope portion of the total ion monitor trace. In those cases where an inadequate ion monitor peak was observed because of low concentrations of pesticides, the scan was initiated at the time estimated for the upslope portion to occur based on previous retention time calculations. As usual, prior recording of the mass spectrum of each pesticide was a pre-

(1) K. E. Elgar, *J. Ass. Offic. Anal. Chem.*, **50**, 1067 (1967).
(2) M. S. Schechter, *Pest. Monit. J.*, **2**, 1 (1968).
(3) J. Robinson, A. Richardson, and K. E. Elgar, 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept. 1966, No. A-75.
(4) B. H. Kennett, ANAL. CHEM. **39**, 1506 (1967).
(5) J. H. Beynon, "Mass Spectrometry and Its Applications to Organic Chemistry," Elsevier Publ. Co., New York, N. Y., 1960 p. 214.
(6) W. H. McFadden and E. A. Day, ANAL. CHEM. **36**, 2362 (1964).
(7) A. B. Littlewood, *Chromatographia*, **1**, 37 (1968).
(8) J. T. Watson and K. Biemann, ANAL. CHEM., **37**, 844 (1965).
(9) D. M. Desiderio, Jr., and T. E. Mead, *ibid.*, **40**, 2090 (1968).
(10) C. C. Sweeley, W. H. Elliott, I. Fries, and R. Ryhage, *ibid.*, **38**, 1549 (1966).
(11) C. G. Hammar, B. Holmstedt, and R. Ryhage, *Experientia*, **24**, 98 (1968).
(12) G. Widmark, L. Bergstedt, and A. Laveskog, Applied Chemistry Division, Pesticides Section, I.U.P.A.C. Conference, Stittingbourne, Kent, U.K., October 1968.
(13) F. J. Biros and A. C. Walker, unpublished data.
(14) W. D. MacLeod, Jr., and B. Nagy, ANAL. CHEM. **40**, 841 (1968).

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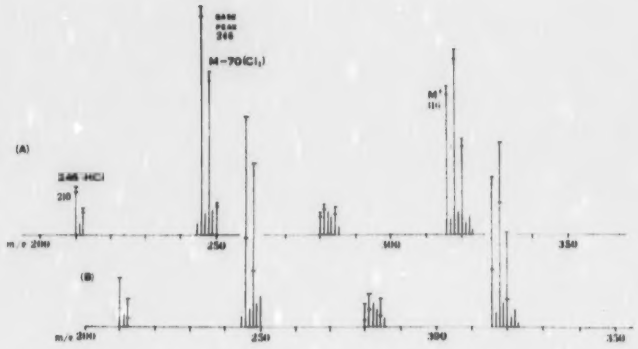

Figure 1. Mass spectra of 3×10^{-4} gram (*a*) and 0.250×10^{-4} gram (*B*) of 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene (p,p'-DDE) injected into a gas chromatograph-mass spectrometer. The spectra were normalized to the base peak. Horizontal bars represent the variation in ion intensity observed from ten recordings of each spectrum

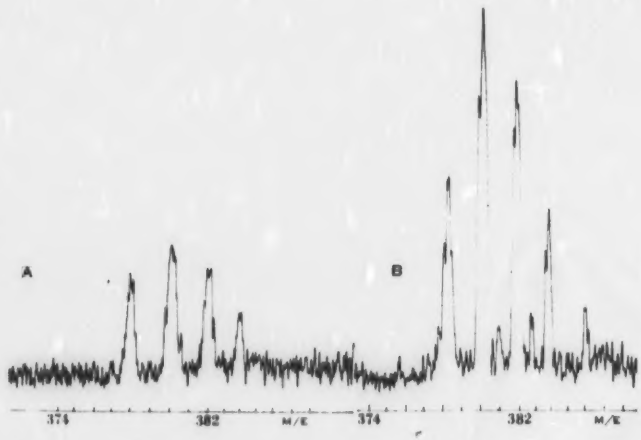

Figure 2. Computer readout of the molecular ion region of 1,2,3,4,10,10-hexachloro-6,7-epoxy - 1,4,4a,5,6,7,8,8a - octahydro - 1,4-*endo*,*exo*-5,8-dimethanonaphthalene (dieldrin) *m/e* 375 to 385, for 50 ng injected into a gas chromatograph–mass spectrometer. Computer input attenuator, IV; verticle range 2^3. *A*. Single scan. *B*, 14 scans-(1 sec/scan)

requisite. An expanded spectrum of 20 to 30 mass units in the range of interest, accurately showing relative intensities in the range, allows the desired *m/e* values to be located without difficulty. The spectrometer is provided with a repeat scan mode facilitating repetitive consecutive scanning of the region of interest. Scans were adjusted in most cases to be of one-second duration. At any scan rate, the mass spectrometer scan was adjusted to be a fraction of a second longer than the computer scan speed to allow synchronization of the start of both mass spectrometer and computer scans.

The time averaging computer used was a Varian C-1024 manufactured by the Technical Measurement Corp., North Haven, Conn. Signal input was provided from the mass spectrometer by a line tee inserted between the electron multiplier preamplifier and filters and the galvanometer amplifiers. The C-1024 trigger was provided through one of the high voltage relay switches (K1) of the mass spectrometer which is energized at the start of each scan and in turn is used to energize the scan stop comparator circuit.

RESULTS AND DISCUSSION

Experimental limitations in precision of ion intensities for scans of samples of 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene (*p,p'*DDE) injected into a combination gas chromatograph-mass spectrometer are shown in Figure 1. Maximum and minimum intensities from ten mass spectra are indicated and show that for low concentrations, the variation in ion intensities may be too great to allow correct recognition of characteristic isotope distribution patterns. In this study, low concentrations of pesticides were examined by combined gas chromatography–mass spectrometry. Certain mass spectral peaks were scanned and recorded after individual scans and computer averaged multiple scans to show the enhancement of sensitivity and ion intensity distribution possible with time averaging techniques.

Identification of chlorinated hydrocarbon pesticide residues by mass spectrometry is greatly facilitated by the occurrence of isotopic distribution patterns for Cl atoms in the molecular ion cluster, as well as for daughter ions and rearrangement ions which contain chlorine atoms (*15*). The distribution pattern results from a 76/24 natural abundance ratio of ^{35}Cl and ^{37}Cl atoms. Thus, characteristic intensity patterns of the *m/e* peaks are obtained and indicate the number of chlorine atoms within the parent ion and/or daughter ions. The statistical distribution of *m/e* peaks for any combination of chlorine atoms is available in the literature (*16*). The mass spectral peaks chosen for each pesticide were those which combined high intensity with characteristic isotopic distribution patterns. This approach was considered adequate in this study to confirm the individual pesticides without resorting to scanning two *m/e* peaks or group of peaks from different areas of the mass spectrum. The mass spectra of dieldrin (*17*), *p,p'*-DDT

(15) T. R. Kantner and R. O. Mumma, *Residue Rev.*, **16**, 138 (1966).
(16) K. Biemann, "Mass Spectrometry, Organic Chemical Applications," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p 66.
(17) J. N. Damico, R. P. Barron, and J. M. Ruth, *Org. Mass Spectrom.*, **1**, 331 (1968).

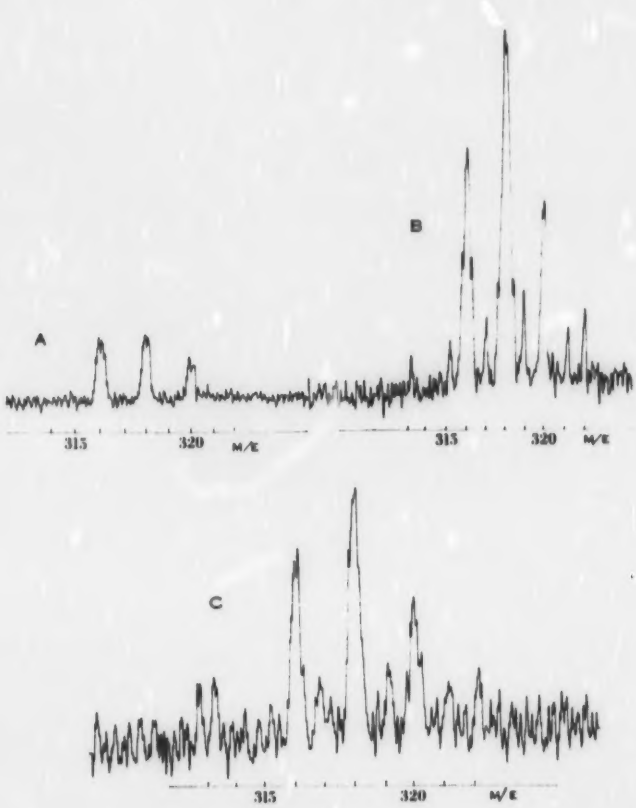

Figure 3. Computer readout of the molecular ion region of 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene (p,p′-DDE) *m/e* 315 to 320, for 1 ng and 5 ng amounts injected into a gas chromatograph–mass spectrometer. Input attenuator and vertical range settings were the same as in Figure 2

A 5 ng, single scan (1 sec)
B 5 ng, 12 scans (1 sec/scan)
C 1 ng, 24 scans (0.25 sec/scan)

and *p,p′*-DDE (*18*) have been reported and the major fragmentation pathways have been assigned.

For dieldrin, the molecular ion cluster was scanned at a concentration level of 50 ng. Generally, five peaks are observable from *m/e* 378 to *m/e* 386, with the *m/e* 380 peak being the most intense and amounting to approximately 40% of the aggregate intensities of the molecular ion peaks. The relative intensity of the *m/e* 380 peak when normalized to the base peak at *m/e* 79 is 4.8%. The base peak in the mass spectrum of dieldrin arises from the decomposition of the norbornadiene ion by loss of CHO (*17*). Figure 2 illustrates the mass spectra in the molecular ion region obtained for a single scan and 14 scans of dieldrin injected into the gas chromatograph. The instrumental conditions for each run are indicated in the figure. The analyzer sector exit slit was maintained at a setting of 4.5 milliinch to allow the maximum number of ions to strike the electron multiplier with minimum sacrifice in resolution. This was a purely arbitrary setting based on preserving a resolution of 400 *m/e*. The effect of the signal averaging is to increase the signal to noise ratio and the distribution of ion intensities by a factor corresponding to the square root of the number of scans. Thus, the mass spectrum of 50 ng of dieldrin injected into the gas chromatograph after a single scan is somewhat distorted and confirmation, at best, would be in some doubt. However, after 14 scans, the signal to noise ratio is of a sufficient order of magnitude, allowing the characteristic five-peak isotope pattern indicating the presence of six chlorine atoms, to be easily recognized.

(18) J. Jörg, R. Houriet, and G. Spiteller, *Monatsh. Chem.*, 97, 1064 (1966).

Examination of a more intense peak in a given mass spectrum obviously can increase the ultimate sensitivity of the technique proportionately. Figure 3 illustrates the mass spectra obtained in scanning the parent ion region of the mass spectrum of *p,p′*-DDE. The characteristic isotopic distribution pattern consists of four peaks at *m/e* 316, 318, 320, and 322. The *m/e* 318 peak, when normalized to the base peak of *m/e* 246 in the mass spectrum of *p,p′*-DDE, has a relative intensity of approximately 82%. In this instance, the parent ion group could be correctly identified at a level of 1 ng of *p,p′*-DDE injected into the gas chromatograph after 24 scans.

The base peak in the mass spectrum of *p,p′*-DDT is found at *m/e* 235 and arises from the loss of a CCl_3 group from the parent ion. Thus, an isotope pattern characteristic of two chlorine atoms is found with three major peaks at *m/e* 235, 237, and 239. The isotopic distribution of this fragment is clearly seen even at levels of 1 ng and 5 ng injected into the gas chromatograph (Figure 4). In this instance, background peaks probably due to column bleed became evident at the 5-ng level. Consequently, the 1-ng scan was repeated immediately after elution of the *p,p′*-DDT with the computer in the subtract mode. As can be seen, the contribution of the background peaks to the spectrum was considerably decreased.

The relative ease with which ion intensities can be reproduced and the low levels of pesticides which can be detected by this technique show that time averaging of mass spectral data may be useful in the confirmation of pesticide residues of known structures in those instances where only a small amount of sample is available. Although further studies of the useful ranges of sensitivity are required, reliable determinations of identity can be made with as little as 50 ng of material in-

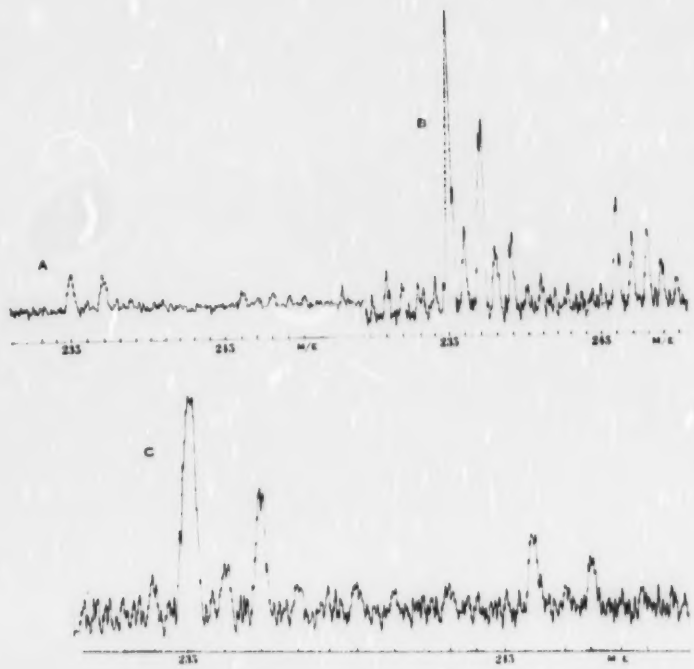

Figure 4. Computer readout of the *m/e* 235 to 250 region of the mass spectrum of 1,1-bis-(*p*-chlorophenyl) 2,2,2-trichloroethane (*p,p'*-DDT) for 1 ng and 5 ng amounts injected into a gas chromatograph–mass spectrometer. Input attenuator and vertical range settings were the same as in Figure 2

A. **5 ng, single scan (1 sec)**
B. **5 ng, 14 scans (1 sec/scan)**
C. **1 ng, 28 scans (0.25 sec/scan)**

jected into a gas chromatograph–mass spectrometer system and possibly with a smaller amount in those instances where the base peak can be examined. Time averaging of mass spectral data leads to a more symmetrically shaped peak; thus peak matching at medium resolution may be a further useful application of this method. Although improved methods of recording mass spectra such as the one described are undoubtedly of some value as an aid to the analyst in recognizing compounds present in pesticide residues, it is possible that a pattern assumed to be the result of a certain isotopic distribution could actually be the result of an entirely fortuitous combination of mass spectral peaks. Further proof of identity may be provided if some additional characteristic patterns derived from the same compound are found in the same mass spectrum.

Improvements in the suggested technique can be made by the interposition of electronic filters to reduce signal noise before it reaches the computer. Preliminary studies in this area support this contention. In addition, the inclusion of an electron capture detector and splitter arrangement in tandem with the molecular separator will allow simultaneous gas chromatographic recording of pesticide effluents with minimum sacrifice of sample at those concentrations where the total ion monitor current is too weak to give rise to a discernible peak.

ACKNOWLEDGMENT

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SHORT COMMUNICATIONS

Correlations between ^{31}P n.m.r. chemical shifts and structures of some organophosphorus pesticides

Several recent publications[1-3] have described the usefulness of high-resolution proton magnetic resonance (n.m.r.) for the elucidation of structure of phosphorus-containing organic compounds used as pesticides. Because phosphorus n.m.r. is playing an ever-increasing role in structure characterization and qualitative identification[4,5] of phosphorus compounds, we have examined ^{31}P n.m.r. spectra of a series of organophosphorus pesticidal compounds. Chemical shifts for thirty-seven organophosphorus pesticides have been measured and correlated with the structures of nine classes of these compounds. Empirical correlations describing the relative effects which the atoms adjacent to phosphorus have on the chemical shifts are given for some of these classes. These correlations between ^{31}P n.m.r. shifts and structure are presented for use in qualitative prediction.

Experimental

The 40.5-MHz spectra were recorded with a Varian HA-100 n.m.r. spectrometer. A capillary tube containing phosphorus oxide, used as an external reference, was inserted into the 5-mm n.m.r. sample tube containing the organophosphorus compound. Analytical standards of organophosphorus pesticides were used. Sample solutions are described in Table I. Chemical shifts were measured by the sideband technique using a Wavetek Multipurpose Oscillator Model No. 116. Positive shifts are upfield from phosphorus oxide; negative are downfield. Compounds are listed by their common or trade names; chemical names and commercial sources for these compounds have been given previously by KENAGA[6].

Results and discussion

High-resolution n.m.r. data provide information about the structure of molecules in two ways. The chemical shift data indicate relative degrees of electron screening of the nucleus, and observation of the electron coupled spin–spin interaction provides additional information on structural relationships of the non-equivalent nuclei in a molecule. In this report only the chemical shifts of some organophosphorus pesticides and the effect on these shifts of the atoms directly bonded to phosphorus are considered. Detailed discussions of spin–spin interactions between phosphorus and proton nuclei of substituent groups will follow in a separate report.

The chemical shifts obtained for thirty-seven phosphorus compounds are given in Table I and are grouped according to the various classes of organophosphorus compounds. For the pentavalent phosphorus, where the atoms adjacent to phosphorus are either sulfur, oxygen, nitrogen, carbon, or a combination of the four, the shifts reported are positive. It can be seen from the data in Table I that the magnitude of the shifts for these compounds decreases in the order phosphates, phosphoramidates, phosphonates, phosphorothioates, phosphoramidothioates, phosphonothioates, and phosphorodithioates.

TABLE I

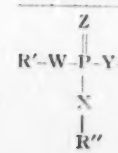
CHEMICAL SHIFTS FOR 37 PHOSPHORUS COMPOUNDS

Compound	*Solvent*	*Chemical shift (p.p.m.)*	*Compound*	*Solvent*	*Chemical shift (p.p.m.)*
Phosphates			*Phosphonates*		
Azodrin	Acetone	118.1	Trichlorfon	Acetone	95.3
Bidrin	Neat	117.6			
Bomyl	Chloroform	118.9	*Phosphorodithioates*		
Ciodrin	Acetone	118.4	Betasan	Chloroform	21.3
Compound 4072	Neat	117.3	Dimethoate (Cygon)	Acetone	15.3
DDVP	Neat	116.1	Disyston	Neat	18.2
Diazoxon	Neat	120.5	Ethion	Neat	20.0
Paraoxon	Benzene	119.7	Guthion	Acetone	18.2
Phosphamidon	Chloroform	117.4	Imidan	Acetone	18.6
Phosdrin	Acetone	118.4	Malathion	Neat	17.5
Ronnoxon	Acetone	117.7	Methyl Trithion	Acetone	15.9
Phosphorothioates			*Phosphoramidothioates*		
Abate	Acetone	46.6	Gophacide	Acetone	56.2
Coumaphos	Neat	50.3	Zytron	Acetone	43.0
Dasanit	Chloroform	50.0			
Diazinon	Neat	52.0	*Phosphoramidates*		
Dursban	Acetone	52.2	Ruelene	Chloroform	104.9
Fenthion	Neat	45.9			
Methyl parathion	Acetone	47.2	*Phosphorotrithioites*		
Nemacide (VC-13)	Neat	49.7	Merphos	Neat	−4.9
Parathion	Benzene	46.0			
Ronnel	Acetone	46.6	*Phosphites*		
Zinophos	Neat	51.2	2,4-DEP	Acetone	−27.1
Phosphonothioates					
EPN	Acetone	28.0			

TABLE II

THE NATURE OF THE ATOMS BONDED TO PHOSPHORUS

Class of compound	*W*	*X*	*Y*	*Z*
Phosphates	Oxygen	Oxygen	Oxygen	Oxygen
Phosphorothioates	Oxygen	Oxygen	Oxygen	Sulfur
Phosphorodithioates	Oxygen	Oxygen	Sulfur	Sulfur
Phosphoramidothioates	Oxygen	Oxygen	Nitrogen	Sulfur
Phosphoramidates	Oxygen	Oxygen	Nitrogen	Oxygen
Phosphonothioates	Oxygen	Oxygen	Carbon	Sulfur
Phosphonates	Oxygen	Oxygen	Carbon	Oxygen
Phosphites	Oxygen	Oxygen	Oxygen	
Phosphorotrithioites	Sulfur	Sulfur	Sulfur	



$R' \neq R'' \neq R'''$ = aryl and/or alkyl.

Two trivalent compounds, a phosphite (2,4-DEP) and a phosphorotrithioite (Merphos), were found to have negative shifts, and the magnitudes of these shifts are consistent with those of similar compounds reported in the literature[7].

The chemical shifts of some phosphorothioates including Diazinon, Parathion, and Ronnel were compared with the shifts of their oxygen analogs, the phosphates Diazoxon, Paraoxon, and Ronnoxon. The differences in the shifts for the phosphorothioates in this series were found to be 68.5, 73.7, and 71.1 p.p.m. upfield with respect to the corresponding phosphates. The only structural difference in these compounds is that the sulfur atom in the P=S bond of the phosphorothioates has been replaced with an oxygen atom (Table II). These upfield shifts are contrary to what one would predict on the basis of electronegativity of the atoms[8] but suggest that the P=O bond has more double bond character than the P=S bond[9]. Replacement of the oxygen atom in the R–O–P bond of the phosphorothioates with sulfur (phosphorodithioates) results in an additional upfield shift, again contrary to expectation on the basis of electronegativity and further suggestive of the relative importance of the contributions of double bonded structures such as the $P=\overset{+}{O}R$ type[9].

A nitrogen-for-oxygen substitution results in a more negative shift[10,11] compared to the phosphate class of compounds. This is shown in Table I for the only phosphoramidate examined (Ruelene) and for the two phosphoramidothioates, Gophacide and Zytron. The exact oxygen and sulfur analogs of these compounds, however, were not available for comparison.

A great deal of theoretical and empirical work has been reported relating the chemical shifts to structures of organophosphorus compounds[1,5,12,13]. The n.m.r. chemical shift data for ^{31}P are presented here in qualitative prediction for organophosphorus pesticides and are shown to be potentially useful in their classification according to structural types.

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1 H. BABAD, W. HERBERT AND M. C. GOLDBERG, *Anal. Chim. Acta*, 41 (1968) 250.
2 L. H. KEITH, A. W. GARRISON AND A. L. ALFORD, *J. Assoc. Offic. Anal. Chem.*, 51 (1968) 1063.
3 L. H. KEITH AND A. L. ALFORD, *Anal. Chim. Acta*, 44 (1969) 447.
4 M. M. CRUTCHFIELD, C. H. DUNCAN AND J. R. VAN WAZER, *Topics in Phosphorus Chemistry, Vol. V*, Interscience Publishers, New York, 1967, pp. 1–74.
5 J. R. VAN WAZER AND J. H. LETCHER, *Topics in Phosphorus Chemistry, Vol. V*, Interscience Publishers, New York, 1967, pp. 169–226.
6 E. E. KENAGA, *Bull. Entomol. Soc. Amer.*, 12 (1966) 161.
7 R. A. Y. JONES AND A. R. KATRITZKY, *Angew. Chem. Int. Ed. Engl.*, 1 (1962) 32.
8 J. A. POPLE, W. G. SCHNEIDER AND H. J. BERNSTEIN, *High Resolution Nuclear Magnetic Resonance*, McGraw-Hill, New York, 1959, p. 349.
9 H. S. GUTOWSKY AND D. W. MCCALL, *J. Chem. Phys.*, 22 (1954) 162.
10 M. L. NEILSEN AND J. V. PUSTINGER, JR., *J. Phys. Chem.*, 68 (1964) 152.
11 J. R. VAN WAZER, C. F. CALLIS, J. N. SHOOLERY AND R. C. JONES, *J. Amer. Chem. Soc.*, 78 (1956) 5715.
12 G. MAVEL, *Progress in NMR Spectroscopy, Vol. I*, Pergamon Press, New York, 1966, pp. 251–373.
13 C. BENEZRA AND G. OURISSON, *Bull. Soc. Chim. France*, (1967) 1825.

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PESTICIDE PUBLICATION

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Environmental Protection Agency

Development of Sampling and Analytical Procedure for Determining Hexachlorobenzene and Hexachloro-1,3-butadiene in Air

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■ Chromosorb 101 efficiently trapped vapors of hexachlorobenzene (HCB) and hexachloro-1,3-butadiene (HCBD) from air at a sampling rate of 3 l./min. No loss of efficiency was noted with sampling times of up to 3 hr. Increasing the sampling rate to 10 l./min. for 1 hr decreased the efficiency by 20%. The method as described has a detection limit of 28 ng of hexachlorobenzene per cubic meter of air.

A recent episode in which the guidelines for hexachlorobenzene (HCB) in meat (0.3 ppm) was exceeded prompted an investigation into the source of the contamination. The grass in pastures around certain chemical plants was found to contain hexachlorobenzene (HCB). Air pollution was considered as one possible source of this contaminant. An efficient, portable, air-sampling device, which would yield HCB in a form amenable to subsequent analysis by electron capture gas chromatography, was needed.

The objective of this study was to develop, evaluate, and field test such a device. An on-going in-house research program designed to improve methodology for determining pesticides in air provided clues to the best approach for resolving this problem (Seiber et al., 1973).

An ethylene glycol impinger system originally described by Miles et al. (1970) and later adapted by Enos et al. (1972) was less desirable for field use than a solid adsorbent trap attached to a portable battery-powered air pump. The solid adsorbent suggested for this work (Seiber, 1973) was Chromosorb 101.

Experimental

Preparation of Chromosorb 101. Chromosorb 101 (60/80 mesh) was extracted for 4 hr using a Soxhlet apparatus charged with 5% acetone in methanol. Six washing cycles per hour proved adequate. A similar wash with hexane was repeated twice. The Chromosorb 101 was dried for 30 min at 70°C. A 1-gram portion was extracted with 20 ml of hexane by shaking in a 50-ml test tube for 20 min on a reciprocating mechanical shaker. A 4-ml aliquot of the supernatant was concentrated to 1 ml and a 5-μl. portion injected into a gas chromotograph for the purpose of establishing the background contribution from the adsorbent. Injection was made into a gas chromatograph containing a 1.5% OV-17/1.95% QF-1 column heated to 150°C and equipped with an electron capture detector. The sensitivity of the gas chromatography system was such that a 50-pg injection of HCB resulted in at least a 50% full-scale deflection with a noise level of less than 2%.

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Table I. Trapping Efficiency for HCB and HCBD on Chromosorb 101

No. of replicates	Air ng/m³	Sampling time and rate	Trapping efficiency, %	Range
		HCB		
5	5560	1 hr, 3 lpm	92.9	88.5–97.5
2	1666	1 hr, 10 lpm	79.0	78.0–80.0
1	1110	1 hr, 3 lpm	97.0	
2	1850	3 hr, 3 lpm	91.6	87.4–95.7
2	370	3 hr, 3 lpm	99.5	98.9–100
		HCBD		
3	4170	1 hr, 2 lpm	100.5	98.0–103

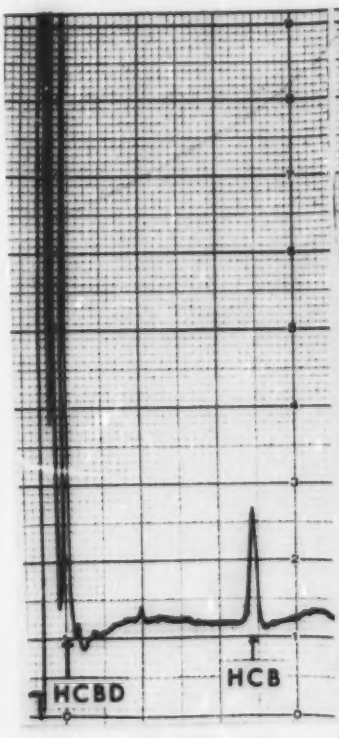

Figure 1. Field sample collected at rate of 2 l./min for 1 hr was equal to 900 ng HCB/m³ air diluted to 40 ml

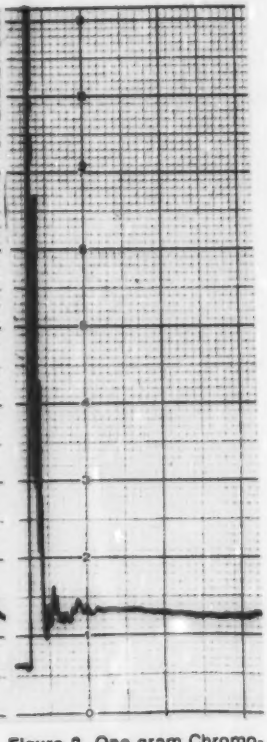

Figure 2. One gram Chromosorb 101 after cleanup; 20 ml concentrated to 5 ml

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Preparation of Collection Tube. A 4-in. length of 10-mm i.d. glass tubing was fire polished at both ends. A crimp was made about 1 in. from one end of the tube to retain a small plug of glass wool. One gram of Chromosorb 101 was placed in the tube followed by another plug of glass wool.

Evaluation of Collection Tube. Known amounts of HCB and hexachlorobutadiene were placed in a U tube and the U tube was connected to the collection tube. After air was drawn through the tubes at a known rate for a specified period of time the Chromosorb 101 was transferred to a test tube containing 20 ml of hexane and shaken for 20 min. Analysis was completed by injecting a 5-μl. aliquot into a gas chromatograph equipped and operated as described above.

Results and Discussion

The results in Table I indicate that a high level of trapping efficiency can be obtained for vapors of HCB and HCBD. HCB was trapped efficiently at a sampling rate as high as 10 l./min (lpm).

For most purposes, a rate of 2 l./min and a sampling interval of 3 hr should be sufficient. Under these conditions and with a preliminary concentration of the extract (4 to 1), a limit of detection of 28 ng/m^3 of air [about 22 parts per trillion (ppt)] can easily be achieved.

HCB was stable when adsorbed on Chromosorb 101 from the vapor phase and stored at room temperature for up to 6 days. This allowed sufficient time to transport samples from the field to the laboratory.

Field evaluation of the sampling device was accomplished by obtaining samples upwind and downwind of a chemical plant manufacturing perchloroethylene. Samples were also taken around a land fill area used by the plant for disposing of waste chemicals.

An air sample taken downwind of the plant contained 990 ng HCB/m^3 while an upwind sample contained 915 ng/m^3. An air sample taken on a dustless day in the vicinity of the land fill contained 18.6 mcg/m^3.

HCBD was also found in the samples described above but it was not quantitated.

The identities of HCB and HCBD in the field samples were confirmed by mass spectrometry.

Figure 1 is a chromatographic tracing depicting both the HCB and HCBD as they appear in a field sample. Figure 2 is typical of the background obtained from Chromosorb 101 when it was prepared and evaluated as described in this method.

Literature Cited

Enos, H. F., Thompson, J. F., Mann, J. B., Moseman, R. F., "Determination of Pesticide Residues in Air," 163rd ACS National Meeting, Boston, Mass., April 1972.

Miles, J. W., Fetzer, L. E., Pearce, G. W., *Environ. Sci. Tech.*, 4, 420–5 (1970).

Seiber, J., Shafik, T., Enos, H. F., progress report, Primate and Pesticides Effects Laboratory, U.S. Environmental Protection Agency, Perrine, Fla., 1973.

Seiber, J., Department of Environmental Toxicology, University of California, Davis, Calif., private communication, 1973.

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INTERFACING OF AN ATOMIC ABSORPTION SPECTROPHOTOMETER WITH A GAS-LIQUID CHROMATOGRAPH FOR THE DETERMINATION OF TRACE QUANTITIES OF ALKYL MERCURY COMPOUNDS IN FISH TISSUE

KEY WORDS: Atomic absorption spectrophotometry, gas-liquid chromatography, alkyl mercury, fish tissue.

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ABSTRACT

A rapid method for the determination of alkyl mercury compounds in fish tissue is presented. The method is based on gas-liquid chromatographic separation, combustion of the column effluent in a furnace and measurement of the resulting atomic vapor by atomic absorption spectrophotometry.

INTRODUCTION

The analysis of mercury in biological and environmental substrates is currently of widespread interest for many investigators. The Hatch and Ott[1] flameless atomic absorption (AA) procedure for the determination of total mercury is probably the most widely used method. Although this method is both specific and sensitive for the determination of the element in trace quantities, it lacks the capability of differentiating among the various inorganic and organic mercurial forms. The differences in the degrees of toxicity between inorganic and organic forms of mercury make it essential to determine not only the total mercury

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concentration but also the relative proportions of the different forms of the element present.

Methods for the determination of organic forms of mercury by gas-liquid chromatography (GLC) using an electron capture detection system have been reported[2-8]. The methods involve a cleanup procedure for separation of the methyl mercury compound from other interferring substances.

Longbottom[16] has recently reported a mercury specific gas chromatographic detector for the determination of dialkyl mercury compounds. We have explored the usefulness of this system using several modifications in order to achieve maximum sensitivity.

This paper describes a GLC-AA procedure for the determination of organic mercury compounds in fish tissue which possesses both sensitivity and specificity and does not require a cleanup step. The method involves separation of the organic mercury compounds by GLC, reduction of the organic mercuries by combustion and detection of elemental mercury by flameless AA.

EXPERIMENTAL

Reagents

Benzene: Purity is such that injection of 10 microliters of a 100 ml aliquot concentrated to 10 ml does not produce interfering responses eluting after the benzene peak upon detection by electron capture.

Hydrochloric acid: Ultra-high purity hydrochloric acid (Ultrex, J. T. Baker Co.) is extracted several times with equal volumes of benzene until injections of the organic layer do not show peaks eluting after the solvent front employing an electron capture detector system.

Cysteine acetate solution: Cysteine acetate solution is prepared according to the procedure described by West[882-4].

Apparatus

Atomic absorption spectrophotometer: Perkin-Elmer model 303, equipped with a 10 cm quartz cell and a Servo/Riter II recorder. The instrument was operated under the conditions recommended by the manufacturer.

Flameless mercury analyzer: Coleman Instruments Model MAS-50 equipped with a 253.7 nm narrow bandpass filter. The cell is disconnected from the air pump. One side of the cell is coupled to the exit of the combustion furnace by means of Teflon tubing (i.d. 1.5 mm). The other side is directed to an activated charcoal trap.

Combustion furnace: Dohrmann Inst. Co., Model S-100, fitted with a 31 x 0.8 cm quartz tube. The furnace is maintained at 780°C with an oxygen gas flow of 25 ml/min. Inlet temperature, 280°C.

Gas chromatograph: Microtek, Model MT-220, equipped with an electron capture detector system, a flame photometric detector (FPD), Malpar Inc., Model 100 A. T., photomultiplier tube, type 9526-S, mercury filter, 253.7 nm, and a U-shaped glass column 1/4" x 6' packed with 5% HIEFF-2AP on Chromosorb WHP, 80/100 mesh (Applied Science Lab, State College, Pa.). Temperature: Inlet 200°C, column 170°C, detector 200°C, transfer lines and outlet block 200°C, Column flow (N_2) 120 ml/min. FPD flame: O_2 and H_2 were mixed in different proportions as described in the results and discussion.

Homogenizer: Sorval Omni Mixer (Ivan Sorval Co., Norwalk, Conn.).

PROCEDURE

Atomic Absorption Detection with the Combustion Furnace

A sample of fish tissue is placed in the homogenizer cup and macerated for 5 minutes at a setting of 4.5. One gram of the homogenized tissue is weighed into a 15 ml glass stoppered centrifuge tube. To the sample tube is added one ml of water and one ml of concentrated hydrochloric acid. The sample is mixed on a Vortex mixer for 3 minutes. Two ml of benzene are added, the tube is shaken vigorously for 5 minutes and centrifuged. The benzene layer is removed, dried over anhydrous sodium sulfate, and a suitable aliquot injected into the gas chromatograph.

After injection of the dried benzene extract, the vent to the combustion furnace unit is opened, thus preventing the solvent from entering the furnace. As soon as the benzene has eluted, the vent is closed and the mercury absorption recorded.

A calibration curve is run with each set of samples by substituting water for tissue and using identical volumes of reagents and benzene. Samples are fortified for recovery studies by adding aliquots of a solution of the appropriate alkyl mercury compounds to the tissue before addition of hydrochloric acid.

Atomic Absorption Detection with Flame Combustion

The preparation of the sample is conducted in the same manner as that described in the above procedure. An aliquot is injected onto the GLC and the solvent is vented. If the flame from the FPD system is used, it is necessary to place a magnesium perchlorate trap between the GLC and the AA in order to absorb the water formed in the O_2/H_2 flame.

Electron Capture Detection

The Westöö procedure for the extraction of Methyl mercury in fish is used[2-4].

RESULTS AND DISCUSSION

The electron capture detector has the sensitivity necessary for trace metal analysis,[9-11] however, it lacks specificity. Recent work by Ross and Shafik[12] has described the use of the flame photometric detector (FPD) system for the determination of trace quantities of chromium in biological samples.

An attempt was made to extend the usefulness of the FPD system to the determination of trace quantities of alkyl mercury compounds. For this purpose, a 253.7 nm filter, quartz heat filters and a photomultiplier tube with a quartz window were incorporated in the FPD, but injections of microgram quantities of methyl mercuric chloride onto the GLC produced no signal. The fact that the FPD afforded no signal response for mercury was not surprising since this detector system is based on the principle of atomic emission, and for many years it has been recognized that mercury is a particularly difficult element to detect using this technique[13]. Mercury was, however, the first element to be determined by AA; the earliest methods had been developed to detect mercury vapor in the atmosphere[14].

The concept of GLC-AA for the detection of volatile metal containing compounds which can be separated by GC techniques is attractive because of the sensitivity and specificity of the AA and the resolving power of the GLC. These features of the GLC and AA systems prompted us to explore the possibility of interfacing the two systems. The combination would allow the alkyl mercuries to

be separated on the GLC column, burned in the oxygen-hydrogen flame of the FPD detector system and detected by diverting the carrier gas to a Perkin-Elmer Model 303 AA spectrophotometer equipped for flameless mercury analysis. A diagram of this system is shown below.

The sample is injected onto the GLC column at A, separated on the column, B, and burned in the flame of the FPD at C. The carrier gas is then diverted through Teflon tubing, D, and through a drying tube, E, which contains magnesium perchlorate. A 10 cm quartz cell, G, replaces the Boling burner in the AA. The mercury vapor is passed through the cell using nitrogen as a carrier gas, and light corresponding to 253.7 nm is passed through the cell. The mercury vapor concentration is detected and recorded at H. With this system, the relatively long transfer lines from the GC to the AA and the drying trap contribute to "dead volume" which in turn results in poor resolution and broad peak shapes.

Replacement of the FPD burner system with a combustion furnace and the AA spectrophotometer with a Coleman mercury analyzer

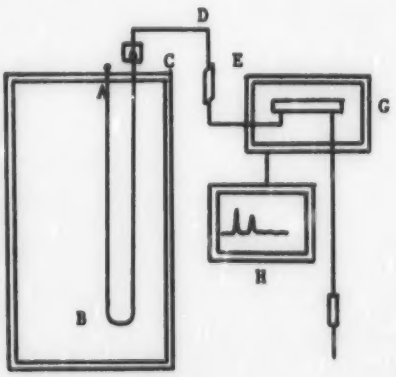

FIGURE 1

eliminates the necessity of the drying tube since water is not formed to any great extent during oxygen combustion. In addition, the smaller AA unit may be placed very close to the exit of the furnace, thus making it possible to use shorter transfer lines. These modifications afforded optimum peak shape and concomitantly, enhanced resolution.

Tracings obtained from injections of benzene extracts of acidified fish homogenates containing 0.1 to 0.5 ppm of mercury are shown in Figure 2.

Injections of reagent blanks and unfortified tissue failed to produce any absorption. The mercury absorption signals are obtained in the form of sharp peaks with some tailing, Figure 3. Similar

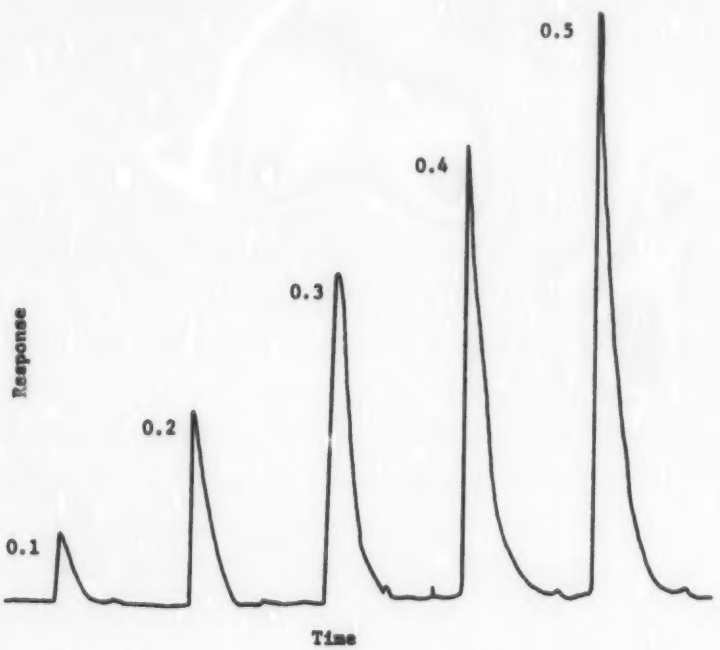

FIGURE 2

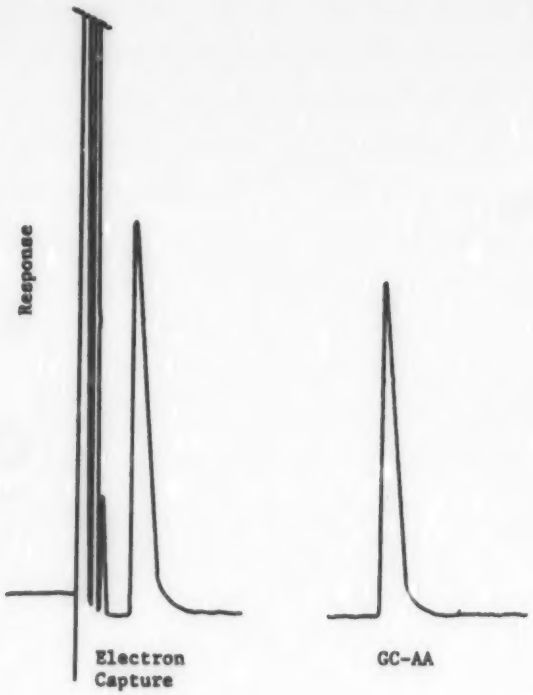

FIGURE 3

peak shapes are observed when samples are analyzed with the electron capture detector using identical GLC conditions and recorder chart speeds. This observation demonstrates that peak shapes in the GLC-AA system are influenced more by the chromatographic system than by the distribution of mercury vapor in the gas stream leading from the GLC unit to the cell of the AA.

Figure 4 shows tracings obtained from a sample containing 0.3 ppm of both methyl mercuric chloride and ethyl mercuric chloride. Good separation of the peaks of these two similar compounds may be observed.

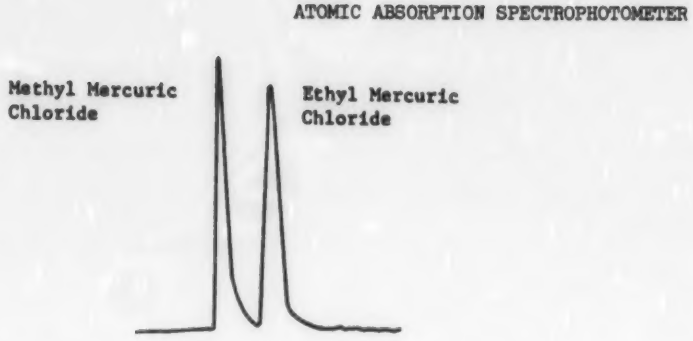

FIGURE 4

Figure 5 demonstrates that the GLC-AA analytical system provides a linear relationship between the concentration of methyl mercuric chloride and peak height up to 45 ng.

The absolute sensitivity, which is defined as the weight of methyl mercuric chloride needed to produce a signal of 1% absorption is 2.5×10^{-11} g. The system as it is presently being operated is approximately twenty times less sensitive than electron capture.

The relative standard deviation (RSD) calculated from 10 injections of 2.4×10^{-9} g of methyl mercuric chloride was 1.7 and

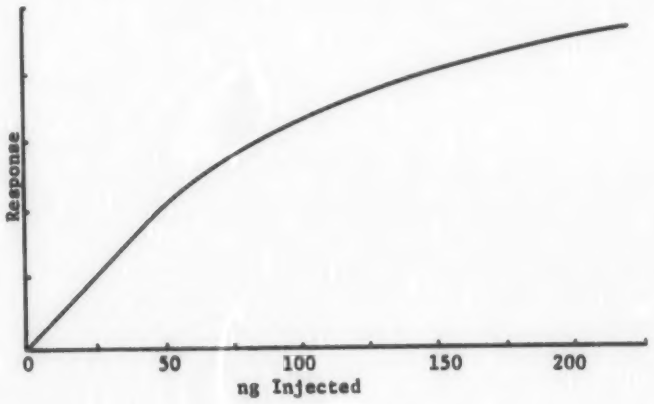

FIGURE 5

4.0 percent for the electron capture and the AA detection techniques respectively.

Table 1 shows results of the recovery studies performed using the GLC-AA system. Table 2, similarly, presents recovery data obtained from the same samples analyzed by electron capture using West88's procedure. Excellent agreement was obtained.

TABLE 1

Added Level (ppm)	Found (ppm)	% Recovery
0.1	0.096	96.0
0.2	0.197	98.5
0.3	0.313	104.3
0.4	0.403	100.8
0.5	0.495	99.0

Recovery of Methyl Mercuric Chloride from Fish Tissue as Determined by the GC-AA System.

TABLE 2

Added Level (ppm)	Found (ppm)	% Recovery
0.1	0.099	99.0
0.2	0.205	102.5
0.3	0.305	101.7
0.4	0.385	96.3
0.5	0.518	103.6

Recovery of Methyl Mercuric Chloride from Fish Tissue as Determined Using West88's Method and Electron Capture Detector.

The partition coefficient of methyl mercuric chloride between benzene and the aqueous phase was considered in calculating the recovery data. All data were obtained by direct comparison with calibration curves prepared in the manner described under "Experimental".

Methods that utilize the electron capture detector for the analysis of alkyl mercury compounds in fish tissue include a cleanup procedure for the organic extract[2-8] to eliminate compounds which may interfere by electron capture.

The method described in this paper involved only one extraction, namely, that of the fish homogenate with benzene. No cleanup is necessary since the flameless atomic absorption technique is much more specific for mercury than the electron capture detector is for the alkyl mercury chloride.

SUMMARY

The method described for the determination of alkyl mercury compounds is based on GLC separation, combustion in a furnace and measurement of the resulting atomic vapor by AA spectroscopy. The procedure is substantially faster and simpler than presently available methods which utilize extensive cleanup procedures and electron capture as the detection system. The sensitivity attainable is adequate for monitoring levels of methyl mercury in tissue[15].

REFERENCES

1. W. R. Hatch and W. L. Ott, Anal. Chem., 40, 2985 (1968).
2. G. Westöö, Acta Chemica Scandinavica, 20, 2131 (1966).
3. G. Westöö, Acta Chemica Scandinavica, 21, 1790 (1967).
4. G. Westöö, Acta Chemica Scandinavica, 22, 2277 (1968).

5. K. Sumino, Kobe J. Med. Sci., 14, 115 (1968).

6. K. Sumino, Kobe J. Med. Sci., 14, 131 (1968).

7. S. Yamaguchi and H. Matsumoto, the Kurume. Med. J., 16, 33 (1969).

8. R. T. Ross, J. G. Gonzalez and L. F. Moore, Paper Presented at the 162nd National Meeting of the American Chemical Society, Washington, D.C., 1971.

9. R. W. Moshier and R. E. Sievers, "Gas Chromatography of Metal Chelates," 1st Ed., Pergamon Press, Oxford, England (1965).

10. W. D. Ross, Anal. Chem., 35, 1596 (1963).

11. E. Bayer, H. P. Muller and R. S. Sievers, Anal. Chem., 43, 2012 (1971).

12. R. T. Ross and M. T. Shafik, Paper presented at the 161st National Meeting of the American Chemical Society, Los Angeles, California (1971).

13. J. A. Dean, Flame Photometry, McGraw Hill, New York, 187,(1960).

14. T. T. Woodson, Rev. Sci., Instrum., 10, 308 (1939).

15. A. C. Kolbye, Science 175, 1192 (1972).

16. J. E. Longbottom, Anal. Chem. 44, 1111 (1972).

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The Determination of 4,4'-Thiodiphenol in Human and Rat Urine as an Indication of Exposure to Low Levels of Abate

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The larvicide Abate® (0,0,0',0'-tetramethyl 0,0'-thiodi-p-phenylene phosphorothioate) has been registered for use in the control of mosquito larvae in nonpotable water. However, there are no specific methods available to determine exposure to Abate in humans and animals.

In an exposure study (1) carried out in Puerto Rico, Abate at a concentration level of 1 ppm was added to drums and cisterns which stored the drinking water for a community of 2,000 people. A total organic phosphorus method was used to analyze the urine from the exposed individuals; the results indicated that equilibrium levels were reached after about 20 weeks of exposure. The alkyl phosphate content in urine dropped rapidly to near control levels at the conclusion of the exposure study.

Abate residues have been determined colorimetrically (2) and gas chromatographically (3, 4, 5). Two of the reported methods are based on the determination of 4,4'-thiodiphenol, the hydrolysis product of Abate, by a spectrophotometric method (2) and a gas chromatographic method (4). All methods are designed to determine Abate in water with the exception of the method reported by St. John and Lisk (6) in which the methylated alkyl phosphate hydrolytic product of Abate was determined in soil extracts.

Metabolism studies have been performed to determine the metabolic fate of labeled Abate when administered orally to rats (7). The fecal (60%) and urinary (40%) routes were the principal means of elimination over a 48 to 72 hour period. Gross radioactivity levels in the blood were highest between 5 and 8 hours after ingestion, then decreased with a half-life of approximately 10 hours. In the feces and in fatty tissue, the radioactivity was due principally to unchanged Abate with minor amounts of its sulfoxide derivative. The principal urinary metabolic products were sulfate ester conjugates of the phenolic hydrolysis products 4,4'-thiodiphenol (24%), 4,4'-sulfinyldiphenol (2.5%) and 4,4'-sulfonyldiphenol (0.05%) (7). Thus the determination of the 4,4'-thiodiphenol in human or rat urine seems to be the best approach to determine low levels of exposure to Abate.

This paper presents a gas chromatographic method which has been developed to determine the extent of human and animal exposure to low levels of Abate.

Commercial sources and trade names are provided for identification purposes only. Their mention does not constitute endorsement by the U.S. Department of Health, Education, and Welfare.

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Vol. 5, No. 4, 1970, published by Springer-Verlag New York, Inc.

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Materials and Methods

Reagents

Tri-Sil concentrate (a 2:1 mixture of hexamethyldisilazane and trimethylchlorosilane), Pierce Chemical Co., Rockford, Ill.

Silica gel, 60-200 mesh, grade 950, Fisher Scientific Co., Pittsburgh, Pa.

Abate and 4,4'-thiodiphenol, both analytical standards, American Cyanamid Corp., Princeton, N.J.

Apparatus and Glassware

Microcolumn, i.d. 7 mm, Catalog No. K-420100, Size 22, Kontes Glass Co., Vineland, N.J.

Crucible holders, glass, top i.d. 25 mm.

Gas chromatograph, MicroTek Model MT 220 equipped with flame photometric detector, sulfur filter, 394 mμ, and a U-shaped aluminum column, 1/4" o.d. x 2', packed with 60-80 mesh Chromosorb W coated with 2.5% E 301 and 0.25% Epon 1001 (w/w). Temperatures: inlet, 190°C; column, 190°C; detector, 135°C; transfer line and outlet block, 200°C. Flow rates (ml/min): nitrogen, 140; hydrogen, 200; oxygen, 40. On-column injection was used for all analyses.

Analytical Procedure

Preparation of Standard Curves

Prepare four standard solutions of the 4,4'-thiodiphenol in chloroform or methylene chloride to contain 0.5, 1, 2, and 4 μg/ml.

Pipet a 1 ml aliquot from each standard solution into a 15 ml centrifuge tube. Add 0.25 ml pyridine and 0.2-0.3 ml Tri-Sil concentrate. Stopper, mix, and heat at 90°C in a heating block for 1 hour or leave overnight at room temperature. Add approximately 2 ml water, follow with 3 ml 1N H_2SO_4, and mix. After complete separation of the two layers, remove the water layer with a disposable pipet. Add approximately 5 ml distilled water and mix. Allow the two layers to separate and remove the water layer. Inject 10 μl of each standard corresponding to 5, 10, 20, and 40 ng of 4,4'-thiodiphenol into the gas chromatograph. Volumes up to 50 μl may be injected. Plot the response or peak height versus concentration on log-log graph paper. The detector response is linear up to 100 ng of the 4,4'-thiodiphenol, using the log-log scale. Eleven nanograms of the derivatized 4,4'-thiodiphenol produce a peak 1/2 full recorder deflection at 6.35×10^{-9} AMP full scale. The minimum detectable amount for 4,4'-thiodiphenol is 5 ng. A typical standard curve obtained in this fashion is shown in Figure 1. The gas chromatogram of the silyl ether derivatives of 4,4'-thiodiphenol, 4,4'-sulfinyldiphenol, and 4,4'-sulfonyldiphenol is shown in Figure 2.

Hydrolysis of the 4,4'-Thiodiphenol Urinary Conjugates

Pipet 1-10 ml of the urine sample into a 25 ml test tube. Slowly add a volume of concentrated HCl equal to half the volume of the urine sample and follow with approximately 0.5 g zinc dust. (The reducing medium is provided to minimize air oxidation of the 4,4'-thiodiphenol.) Place a reflux condenser on the tube and cool the condenser with circulating ice-cold water. Heat the tube in a heating block maintained at 120°C for 1 hour. Allow the tube to cool, wash down the sides of the condenser with about 2 ml of chloroform or methylene chloride, disconnect the tube, remove from the condenser, and proceed to the extraction and derivatization step.

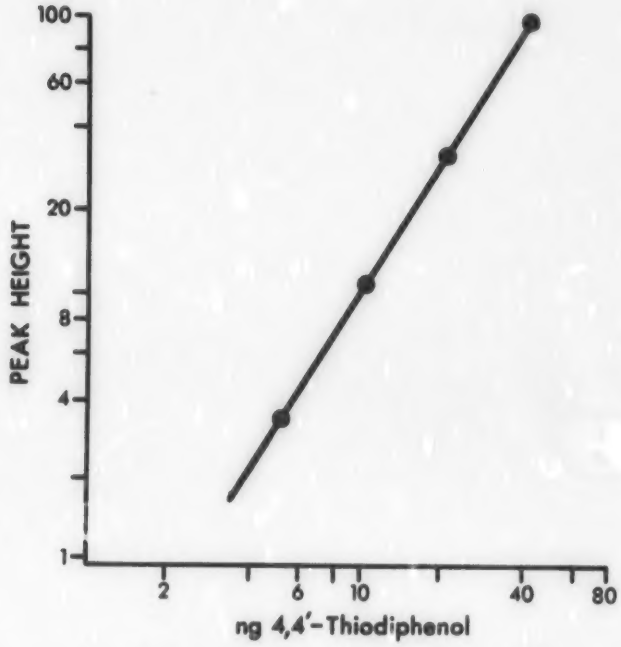

Figure 1. Standard curve of 4,4'-thiodiphenol TMS.

Extraction and Derivatization

Extract the hydrolyzed urine four times with a total volume of 10 ml chloroform or methylene chloride to remove 4,4'-thiodiphenol. Centrifuge the samples during the extraction if emulsions persist. Combine the extracts and transfer to a crucible holder containing about 25 g of anhydrous sodium sulfate and a loose plug of silanized glass wool. Collect the eluate in a 15 ml centrifuge tube.

Concentrate the extract to a volume of 1 ml with the aid of a gentle stream of clean, dry nitrogen and a heating block maintained at 50°C. Proceed with the derivatization step by adding the pyridine and Tri-Sil concentrate and wash to remove the excess reagents as mentioned under the preparation of standard curve.

Chromatographic Removal of Interfering Materials in Urine

Place a small loose plug of silanized glass wool in the tip of the micro-column. Slowly add 1.0 g of 60-200 mesh silica gel, gently tapping the column to insure uniform packing. Add 3 g of granular anhydrous sodium sulfate to the top of the column. Wash the column with 10 ml methanol; then condition the columns in a 130°C oven overnight before using.

Transfer the 1 ml volume of silanized chloroform extract quantitatively to the silica gel column and elute with 10 ml chloroform, collecting the eluate in a 15 ml centrifuge tube. Concentrate the eluate to a volume of 1.0 ml with the aid of a gentle stream of clean, dry nitrogen and a heating block maintained at 50°C. Inject aliquots up to 50 µl in the gas chromatograph.

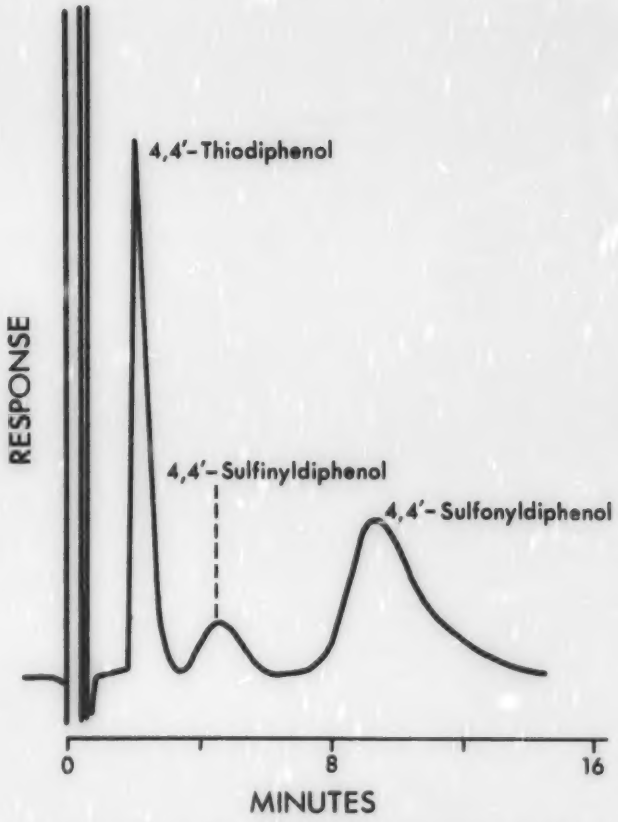

Figure 2. Chromatogram of the silyl ether derivatives of 10 ng 4,4'-thiodiphenol, 4,4'-sulfinyldiphenol undetermined, and 32 ng 4,4'-sulfonyldiphenol.

Recovery of 4,4'-Thiodiphenol from Human Urine

Five 10 ml aliquots of a human urine sample were fortified with 4,4'-thiodiphenol at the levels of 0.02, 0.05, 0.10, 0.50, and 1.0 ppm. The fortified samples were hydrolyzed, extracted, and derivatized, and the interfering materials were removed using the microcolumn cleanup described above.

The Determination of 4,4'-Thiodiphenol in Rat Urine

Two female Sprague-Dawley rats weighing 242 and 238 g were dosed by gavage with 9.6 mg of Abate (1/10 the LD_{50}) dissolved in peanut oil containing traces of ethanol. The dose was administered daily for three consecutive days. The animals were maintained in a stainless steel metabolism cage, and the urine was collected in a manner which minimized fecal contamination. Timed urine samples were collected before exposure and at various intervals thereafter; all urine samples were frozen until analyzed for 4,4'-thiodiphenol. Results are expressed as micrograms excreted per day.

Results and Discussion

The results of the analysis of the human urine sample fortified with 4,4'-thiodiphenol at the levels from 0.02 to 1.0 ppm using the suggested procedure are shown in Table I. The recoveries ranged from 75 to 87%, and levels as low as 0.02 ppm 4,4'-thiodiphenol were detectable.

TABLE I

Recovery of 4,4'-Thiodiphenol Added to 10 ml of Human Urine

Level of Fortification, ppm	Amount Recovered, ppm	Recovery, %
0.02	0.015	75
0.05	0.039	78
0.10	0.079	79
0.50	0.400	80
1.00	0.870	87

Appreciable amounts of 4,4'-thiodiphenol were found in the urine of rats dosed three times with a concentration of Abate equivalent to 1/10 the LD_{50}. The rate of urinary excretion of the phenol is shown in Figure 3; the results are expressed as micrograms excreted per day. Levels

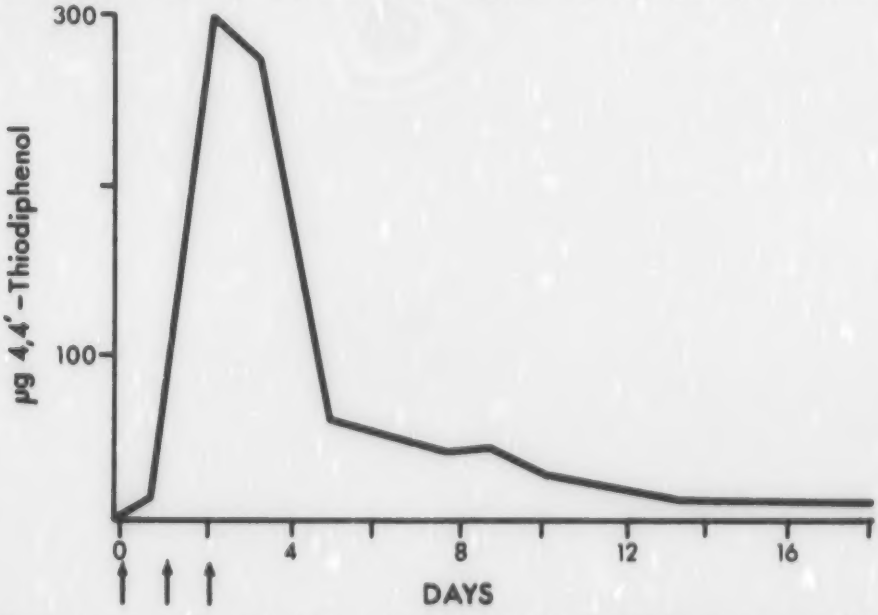

Figure 3. The rate of urinary excretion of 4,4'-thiodiphenol of rats fed three consecutive 1/10th LD_{50} dosages. Arrows indicate the time of dosage.

as high as 0.20 ppm of the 4,4'-thiodiphenol were still being excreted in the urine two weeks following the last dose. These data indicate that Abate or Abate metabolites may be stored in tissues of the rat. The possibility of storage has been reported by Laws et al. (8). These workers observed that the organic phosphorus level in human urine decreased slowly after the dosage was stopped and was still above control levels three weeks after the last exposure.

Small amounts of 4,4'-sulfinyldiphenol and 4,4'-sulfonyldiphenol residues in urine can be determined by extracting the acid-hydrolyzed sample with diethyl ether instead of chloroform. The ether extract is evaporated to dryness, redissolved in 1 ml chloroform, silanized, washed, and injected in the gas chromatograph without the silica gel cleanup step. The difficulty in obtaining a pure 4,4'-sulfonyldiphenol standard can be conveniently circumvented by oxidizing 4,4'-thiodiphenol with hydrogen peroxide; 10 to 20 µg samples of 4,4'-thiodiphenol can be oxidized quantitatively to the 4,4'-sulfonyldiphenol. Attempts to prepare a pure 4,4'-sulfinyldiphenol were unsuccessful.

The hydrolysis step, which includes a zinc and HCl medium maintained at 100°C, does not reduce either the 4,4'-sulfinyldiphenol or the 4,4'-sulfonyldiphenol residues present in the urine. Furthermore, these reagents serve to prevent the oxidation of the 4,4'-thiodiphenol. For this reason, a reducing medium is used in the hydrolysis step and solvents are evaporated with a stream of nitrogen instead of air.

The lower limit of detection using the suggested method and cleanup developed in this paper is 0.02 ppm.

Summary

A method for the determination of Abate in water has been modified to determine the 4,4'-thiodiphenol in human and rat urine. The method involves acid hydrolysis of conjugated residues, extraction, silylation, cleanup, and gas chromatography using the flame photometric detector equipped with a sulfur filter. The method can be used for the determination of the other Abate metabolites, 4,4'-sulfinyldiphenol and 4,4'-sulfonyldiphenol. The recovery of the 4,4'-thiodiphenol from fortified human urine was 75-87%. The minimum detectable amount is 0.02 ppm.

References

1. LAWS, E.R., Jr., SEDLAK, V.A., MILES, J.W., JOSEPH, C.R., LACOMBA, J.R. and RIVERA, A.D., Bull. World Health Organ. 38, 439 (1968).

2. BLINN, R.C. and PASARELA, N.R., J. Agr. Food Chem. 14, 152 (1966).

3. DALE, W.E. and MILES, J.W., J. Agr. Food Chem. 17, 60 (1969).

4. SHAFIK, M.T., Bull. Environ. Contam. Toxicol. 3, 309 (1968).

5. WRIGHT, F.C., GILBERT, B.N. and RINER, J.C., J. Agr. Food Chem. 15, 1038 (1967).

6. ST. JOHN, L.E., Jr. and LISK, D.J., J. Agr. Food Chem. 16, 408 (1968).

7. BLINN, R.C., J. Agr. Food Chem. 17, 118 (1969).

8. LAWS, E.R., Jr., MORALES, F.R., HAYES, W.J., Jr. and JOSEPH, C.R., Arch. Environ. Health, 14, 289 (1967).